

Bioactivities of Wood Polyphenols: Antioxidants and Biological Effects

By

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Dedication

I dedicate this document to my husband for his support, trust, motivation and being with me at every step of this journey.

LIST OF ABBREVIATIONS

SPW	Seedling date palm wood
OPW	Old date palm wood
OW	Oak wood
QW	Quebracho
PW	Pine wood
BW	Banana bark
MAW	Maple wood
MALDI	Matrix Assisted Laser Desorbed Ionization
DW	Dry weight
LDL	low-density lipoprotein
SP	Soluble phenolic
IPB	Insoluble-bound phenolic
TBARS	2-Thiobarbituric acid reactive substances

FRAP	Ferric reducing antioxidant power
CID	Collision-Induced Dissociation
DHB	2,5- dihydroxybenzoic acid
RDA	retro-Diels Alder reaction
CW	Cell wall
VRL	Virgin Released Lignin
DBE	Double Bond Equivalent

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ABSTRACT

The phenolic and polyphenolic compounds present in date palm, oak, quibracho, banana, and pine wood play a crucial role in the development of natural antioxidants. This study provides structural information on the phenolic compounds isolated from wood obtained from a series of old date palm (OPW), seedling date palm (SPW), oak (OW), quibracho (QW), banana (BW), and pine (PW) and their associated products. The total phenolic content (TPC), total flavonoid content (TFC), tannin content (TC) as well as the antioxidant activity of wood extracts was studied by employing *in vitro* assays using the free radical scavenger 1,1-diphenyl-2-picrylhydrazyl (DPPH) and iron chelation methods. The antioxidant activity and phenolic content of SPW extract, as determined by the DPPH assay, TPC and TC were higher than those of the other wood extracts. Analysis of wood extracts revealed a high amount of phenolics and tannins suggesting a possible role of wood phenolics as a source of natural antioxidants. These studies clearly indicate that the SPW had a significant antioxidant activity. In addition, the antioxidant capacity of soluble- and insoluble-bound phenolics of wood extracts was examined, as well as their bioactivities such as inhibition of oxidation of LDL cholesterol and DNA strands breakage. The results so obtained also confirmed the fact that antioxidant potential and oxidation inhibitory activities were much higher for the SPW compared to other wood extracts. Furthermore, in this study, the date palm wood was used to generate smoke that was subsequently used to prepare smoked salmon, using maple wood as a commercial standard. Prominent volatile compounds released during the smoking process were adsorbed using solid-phase microextraction (SPME) and were then identified by gas chromatography–mass spectrometry (GC–MS). The oxidative stability of the smoked salmon was evaluated in terms of development of thiobarbituric acid reactive substances (TBARS). Regardless of the wood type,

volatile compounds were mainly methoxyphenols, with the highest contribution from eugenol, followed by guaiacol and their corresponding derivatives.

The phenolic composition of OPW, SPW, OW, QW, BW, and PW were studied using conventional analytical methodologies. In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI- TOF/TOF-MS) and tandem mass spectrometry (MS/MS) analyses were used to examine the structures of various phenolics, tannins, and lignin compounds. The results showed that OPW, SPW, OW, QW were rich in the classes of compounds tested. Both condensed tannin and hydrolyzable tannins were present in high amounts in SPW, OPW, and OW and their phenolics present showed the highest antioxidant values compared to QW, BW, and PW.

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CHAPTER 1

INTRODUCTION

Phenolics are found abundantly in both edible and nonedible plants, and they are reported to have multiple biological effects, including antioxidant activity. Crude extracts of plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers, consumers and related industries as the trend of the future is moving toward functional food with specific health effects (Löliger, 1991).

Phenolic compounds can be classified as soluble (SP) and insoluble-bound (IBP). The soluble forms are simply extracted using organic solvents and occur in both the free and soluble ester forms (Antolovich *et al.*, 2000). Bound phenolic compounds are typically involved in cell wall structure and require acid, base or enzyme hydrolysis to release them from the cell wall matrix. The level of phenolics in plant sources also depends on cultivation techniques, cultivar, growing conditions, extraction methods and extraction temperature, ripening process / stage, as well as processing and storage conditions (Ongphimai *et al.*, 2013).

Potential sources of phenolic compounds are vegetables, fruits, leaves, oilseeds, cereal crops, wood barks and roots, spices and herb (Ramarathnam *et al.*, 1995). Phenolics such as flavonoids, phenolic acids, stilbenes, tannins, lignans, and lignins are especially common in leaves, flowering tissues, and woody parts such as stems and barks (Larson, 1988). They are important

in the plant for normal growth development and defense against infection, herbivory and injury (Kähkönen *et al.*, 1999). Wood, as such and as a source of phenolics, has always been considered as an important material for humans. Its usefulness has not diminished because of the multiplicity of its uses (Saha *et al.*, 2013). Wood like other plant biomass is essentially a composite material constructed from oxygen-containing organic polymers, which include naturally present cellulose, hemicellulose, and lignin that represent about 30-40, 20-30, and 25-30%, respectively (Fernandez de Simon *et al.*, 1996). Woody plants are divided into two principal groups in terms of their anatomy; these are called softwood and hardwood. In chemical terms, the difference between hardwood and softwood is reflected in the composition of the constituent lignin. Hardwood lignin is primarily derived from sinapyl alcohol and coniferyl alcohol, while softwood lignin is mainly derived from coniferyl alcohol (Boerjan *et al.*, 2003). Several studies have been carried out on the extractives from wood, especially on phenolic compounds. Measurements of extracted wood phenolics and their antioxidant activity have become important tools to understand the values of wood species from a health point of view in biological activities (Diouf *et al.*, 2006). Well-known polyphenols and lignin have been extracted from wood, straw, and bark, which may also be obtained from compressing wood to procure wood oil from wood pulping (Dykes and Rooney, 2006). Wood phenolics have been identified from various plant trees such as olive (Perez-Bonilla *et al.*, 2006; Conde *et al.*, 2009), nuts (Molyneux *et al.*, 2007), oak (Aloui *et al.*, 2004; Fernández de Simón *et al.*, 2014; Gallego *et al.*, 2012; Mullen *et al.*, 2011; McPherson *et al.*, 2014), pine (Conde *et al.*, 2013; Esteves *et al.*, 2011), and wheat straw (Huijgen *et al.*, 2012; Wild *et al.*, 2012).

In this study, six different types of wood were examined for their antioxidant activity and total phenolics content, including phenolic acids, flavonoids, tannins (hydrolysable and condensed

tannins), and lignin. The samples examined included the seedling date palm wood (*Phoenix dactylifera*) (Chao and Krueger), aged 4 years (SPW), and an old date palm wood (*Phoenix dactylifera*), aged 28 years (OPW) (Chao and Krueger), oak (*Quercus robur L.*) wood 18 years (OW) Rozas (2005), pine (*P.strobus*) wood 17 years (PW) (Suga *et al.*,1993), quebracho (*Schinopsis balansae*) wood 17 years (QW) (Vivas *et al.*, 2004) and banana (*Musa acuminata*) bark 1 year (BW)(Anal *et al.*, 2014).

Wood can also be used for production of wood smoke. Wood smoke can retard oxidative rancidity in smoked foods. Phenols are the major contributors to wood smoke aroma, but other compound classes are also important. Since different species of trees have different ratios of components, various types of wood impart a different flavor to food. Analytical data have previously been provided for lignin-specific methoxyphenols in smoke from forest biomass burning (Kjallstrand *et al.*, 1998; Kjallstrand *et al.*, 2000). Methoxyphenols were early identified as prominent thermal degradation products of wood (Faix *et al.*, 1990) and as semi-volatile components of wood smoke particles (Hawthorne *et al.*, 1989).

The role of phenolic-based wood smoke volatiles is discussed in this thesis. Compositional differences between smoke date palm wood (SPW) and maple wood (MAW) with respect to their smoke volatile releasing mechanism have been also described. Various phenols (guaiacol, 4-methylphenol, 2,6-dimethoxyphenol) have been described as possessing smoky aromas. This work also focused on the phenolics identified in wood smoke which exhibited strong antioxidant effects. The effect of the deposition of phenolic compounds from date palm and maple wood was evaluated for smoking time, smokehouse temperature, and initial temperature of fish fillets.

In this study, smoke from palm wood (SPW) was applied to salmon, as a preservation method to compare its phenolics content with other commercial wood (particularly maple) that is

commonly used in Canada for smoking. Overall, the woods that were examined in this study were found to provide excellent and promising sources of phenolics and lignin as natural antioxidants; and consequently, help in the reduction of by-products as discards.

The last objective of the present study was to examine the potential of using wood extracts as an effective source of natural antioxidants and, specifically, to compare the six different varieties of woods with respect to their total phenolic content and antioxidant activity. The possible structures of some phenolic compounds in different wood extracts were also determined using soft ionization mass spectrometry and high-energy collision induced dissociation tandem mass spectrometry analysis using a MALDI-TOF/TOF-MS/MS instrument.

Finally, a separate introduction of the structure of lignins has been provided in Chapter 5 as its content represents a manuscript send for publication consideration to the journal of *Rapid Communication in Mass Spectrometry*.

CHAPTER 2

LITERATURE REVIEW

2.1. Historical Background of wood

When woody plants covered most of the earth's surface, they provided not only food, warmth, and shelter, but many of the needs of defense, medicine, culture, and simple pleasure as tools for sports, art and other applications. Some needs were readily satisfied by using minor forest products such as resins for caulking boats, for use in torches, or for providing rigidity in the attachment of spear, arrow, or axe-heads to the shaft before the joints were finally with fibrous material (Hillis, 1989). Other materials, such as tannins extracts, were used as astringent compounds and also for treatment or other purposes.

Most wood extracts and exudates were probably used locally as biodegradable material, but little evidence remains of their use and their complex nature largely prevented identifying their origin. In addition, many uses of minor forest products have decreased considerably in recent years, hence the history of their use could never be complete

2.1.1. Major uses of wood extractives

Extractives are considered as the secondary metabolites of wood cells that can usually be procured with neutral solvents. Exudates are extracellular secondary metabolites that are formed by trees growing under certain conditions or after injury by fire, insect, fungal, or mechanical damage (Taylor, 1984). In many cases the exudates and the wood and the bark extractives of the same species can have significantly different compositions, but they frequently were used for the

same purposes such as soaking hides in crushed tissues rich in tannins, or by using woods naturally containing toxic components as arrowheads or in spears (Nunez *et al.*, 1986).

2.1.2. *The use of woods*

The Chinese have appreciated the durability of wood for many centuries. The Imperial places were built almost entirely on *Persea nan-mu* (Lauraceae). The high demand for this wood showed that these timbers are long-lasting and durable. This indicates that careful observations lead to the selection of this wood. Similar buildings exist in Japan. Also, the durable timbers of wood have been used for buildings, boats and coffins for many countries and have survived microbiological and insect attack for centuries (Hillis, 1987). Historical records show that some bark, wood and/or tree extracts are used in major industrial products such as perfumes, dyes, gums, lacquers, varnishes, rubber, medicinal purposes, and leather production (Rowe, 1989).

2.2. Woody plants and their chemical composition

Primary shoots of trees and other plants grow in response to inherent patterning mechanisms such as apical dominance and environmental factors like light and gravity to establish a branching architecture that is optimal for growth and reproduction (Felten and Sundberg, 2013; Scurfield, 1973). Perennial woody plants dominate many natural land ecosystems. Their major difference to annual herbs is their long lifecycle that, in trees, may span several centuries, encompassing germination, seedling, juvenility, maturity, senescence and finally death. To sustain their competitiveness through the course of their long life cycle, trees have acquired multiple adaptation strategies that, as a whole, can only rarely be found in annual plant species (Fromm, 2013).

The chemical composition of wood varies from species to species, but is approximately 50% carbon, 42% oxygen, 6% hydrogen, 1% nitrogen, and 1% other elements (mainly calcium, potassium, sodium, magnesium, iron, and manganese) by weight (Barette and Mayer, 1996). Wood also contains sulfur, chlorine, silicon, phosphorus, and other elements in small quantities. Aside from water, wood has three main components: Cellulose, a crystalline polymer derived from glucose, constitutes about 41–43%. Next in abundance is hemicellulose, which is around 20% in deciduous trees but near 30% in conifers. Its monomer units are linked in an irregular manner, in contrast to the cellulose. Lignin is the third component present at around 27% in coniferous wood and 23% in deciduous trees. Lignin confers the hydrophobic properties reflecting the fact that it is based on aromatic rings. These three components are interwoven, and direct covalent linkages exist between the lignin and the hemicellulose components.

Wood like other plant biomass is essentially a composite material constructed from oxygen-containing organic polymers (Mohan *et al.*, 2006). Woody plants have two principal groups in terms of their anatomy; one is called softwood, which commonly has needle-like leaves and naked seeds. The other is known as hardwood, which has broad leaves and enclosed seeds (Figure 2.1). Both the hardest and softest woods on the earth are hardwoods; however, hardwoods are generally denser and harder than softwoods and they are different in external and structural appearance (Smith *et al.*, 2003). In chemical terms, the difference between hardwood and softwood is reflected in the composition of their constituent lignin. Hardwood lignin is primarily derived from sinapyl alcohol and coniferyl alcohol. Softwood lignin is mainly derived from coniferyl alcohol (Boerjan *et al.*, 2003).

Besides providing lumber, plywood, and veneer, pulp board and paper, wood has always been an important material for humans. Its usefulness has not diminished because of the multiplicity of its uses. Heartwood of some tree species has high extractive content. In addition to their contribution to wood colour and odour, they are usually rich in polyphenols, which have many favourable effects on human health such as inhibiting the oxidation of low-density lipoproteins (Frankel *et al.*, 1993), thereby decreasing the risk of heart diseases. They have anti-inflammatory (Albishi *et al.*, 2013b) and anti-carcinogenic properties. Thus, measurements of polyphenols and antioxidant activity of wood extracts have become important tools to understand the value of wood species from a health point of view. Well-known examples may include gallic acid, tannic acid, tannins in general, sequerins, and lignin. Polyphenols can be extracted from wood, straw, and bark, which may also be obtained from compressing wood to obtain wood oils and also from wood pulping (Dykes and Rooney, 2006). Extractives of woods have been found to have antioxidant and other biological activities (Diouf *et al.*, 2006). As essential source of phenolic contents, wood phenolics have been identified from various trees such as olive (Perez-Bonilla *et al.*, 2006; Conde *et al.*, 2009), nuts (Molyneux *et al.*, 2007), oak (Aloui *et al.*, 2004; Fernández de Simón *et al.*, 2014; Gallego *et al.*, 2012; Mullen *et al.*, 2011; McPherson *et al.*, 2014), pine (Conde *et al.*, 2013; Esteves *et al.*, 2011), wheat straw (Banoub, 2014; Huijgen *et al.*, 2012; Wild *et al.*, 2012). Pine, spruce, and larch are classified as softwood; oak, ash, beech, cottonwood, and palm are classified as hardwood.

As mentioned earlier, lignin is an important constituent of wood representing 25-30% of its total weight. Lignin is primarily found within the secondary cell wall and is largely responsible for the stiffness of dry wood; this is in contrast with normal cellulosic plants. The UV portion of sunlight causes photo-degradation of lignin. Degradation initiates with the formation of low

molecular weight polyphenol substances which is evidenced by the darkening of the soluble wood surface exposed to sunlight (Bulian and Graystone, 2009). The subsequent bleaching of such soluble compounds is caused by water in its different forms (condensation, rain, snow, or frost).

There are many extraneous substances present in wood including both inorganic (oxides, salt) and organic compounds, which vary among the different wood species in quantities that range from 5 to 30%. The organic compounds are known as extractives that are extracted from wood by using an appropriate solvent. Although some extractives perform important metabolic functions, others are waste products and their presence in heartwood confers useful protective properties particularly decay resistance. Extractives can be divided according to their chemical composition into three major subgroups: aromatic phenolic compounds, aliphatic compounds (fats and waxes), and terpenes and terpenoids.

The phenolic compounds are second only to carbohydrates in abundance in wood. They are principally found in heart wood and responsible for their deeper colour and decay resistance. In that respect, it is important to distinguish between sapwood and heartwood. Sapwood is the living, outermost portion of a woody stem or branch, while heartwood is the dead, inner wood, which often comprises the majority of a stem's cross-section. You can usually distinguish sapwood from heartwood by its lighter color.

The phenolic compounds can be sub-divided into four groups, which are, lignans, stilbenes, flavonoids, and tannins (Fengel and Wegener, 1989; Hill, 2006). Lignans are stable and colourless, in contrast to stilbenes that darken in the presence of light; darkening of pine is a good example caused by pinosylvin that can be reacted with some aromatic amines as a test for the presence of heartwood in pine. Flavonoids in woods contain a dibenzyl propane unit and are

the principal coloring material in trees, plants, and flowers. They are mostly found in the heartwood of trees. Polymeric flavonoids are also known as condensed tannins. These tannins will be described later in this chapter. Another type of reactive polyphenol contains an unusual seven-carbon ring known as a *tropolone*; thujaplicins are one of the best-known examples. They can chelate with iron to give black/blue staining (Hill, 2005; Bulian and Graystone, 2009).

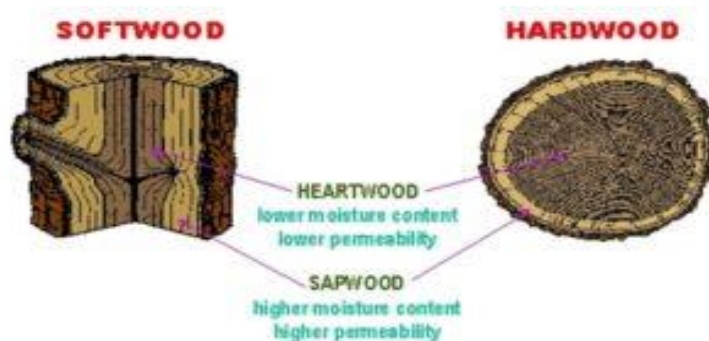


Figure 2.1. Principal groups of woody plants (Hardwood and Softwood). Adapted from (www.google.ca)

2.3. The chemistry of wood

Wood is a composite material composed of fibers of cellulose (40-50%) and hemicellulose (20-30%) held together by a third substance called lignin (25-30%). Although these substances can be considered as mainly macromolecular wood components, other polymeric substances are also present in small amounts as starch and pectin derivatives.

2.3.1. Cellulose

Cellulose is an organic polymer belonging to the polysaccharides family. It is a linear macromolecular built up of a simple sugar, namely glucose (Figure 2.2) units, attached to each other via beta-linkages. Cellulose is the main constituent of the cell walls of wood fibers. It is a highly polar substance due to the presence three-OH groups for every structural unit. These

chemical groups give cellulose a strong affinity for water but cellulose can only absorb water without dissolving in it. Depending on the climatic conditions, air humidity, in particular, cellulose can absorb or release water from the environment with important consequences for the products made of wood.

2.3.2. Hemicellulose

Hemicellulose is another key component of wood secondary cell wall. It is usually embedded among cellulose microfibril bundles, as shown in Figure 2.2. Hemicellulose contains glucose, sugars and derivatives. There are two common types of the major components of hemicellulose: xylans for hardwood and glucomannans for softwood. The main hemicellulose polysaccharides are xylose- β -(1, 4) mannose- β -(1, 4) glucose- α -(1, 3) galactose (Figure 2.2).

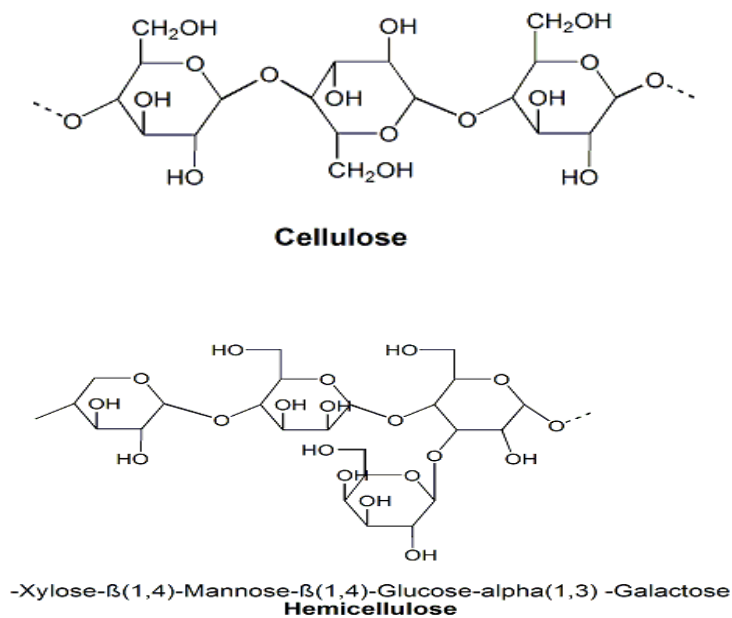


Figure 2.2. The chemical structure cellulose and hemicellulose

2.3.3. Lignin

In addition to cellulose and hemicellulose, another important component of wood cell wall is lignin. Lignin is a phenolic substance consisting of an irregular array of variously bonded hydroxy- and methoxy-substituted phenylpropane units. The precursors of lignin biosynthesis are *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) (Figure 2.3). S is a minor precursor of softwood and hardwood lignins; S is the predominant precursor of softwood lignin; S and G are both precursors of hardwood lignin (Mu *et al.*, 2018). These alcohols are linked in lignin by ether and carbon–carbon bonds. Figure 2.4 is the schematic structure of lignin meant to illustrate the variety of structural components. Please note, as mentioned in Chapter 1 (Introduction), a full introductory section has been provided in Chapter 5 to detail specifications of wood lignins. Thus, structures characteristics of wood lignins in Chapter 5 represents a manuscript that has been sent for publication consideration to the journal of *Rapid Communication in Mass Spectrometry*.

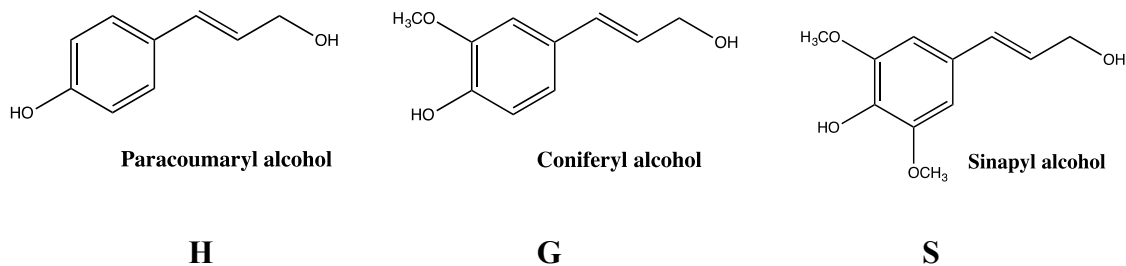


Figure 2.3. The three monomers building lignin in nature

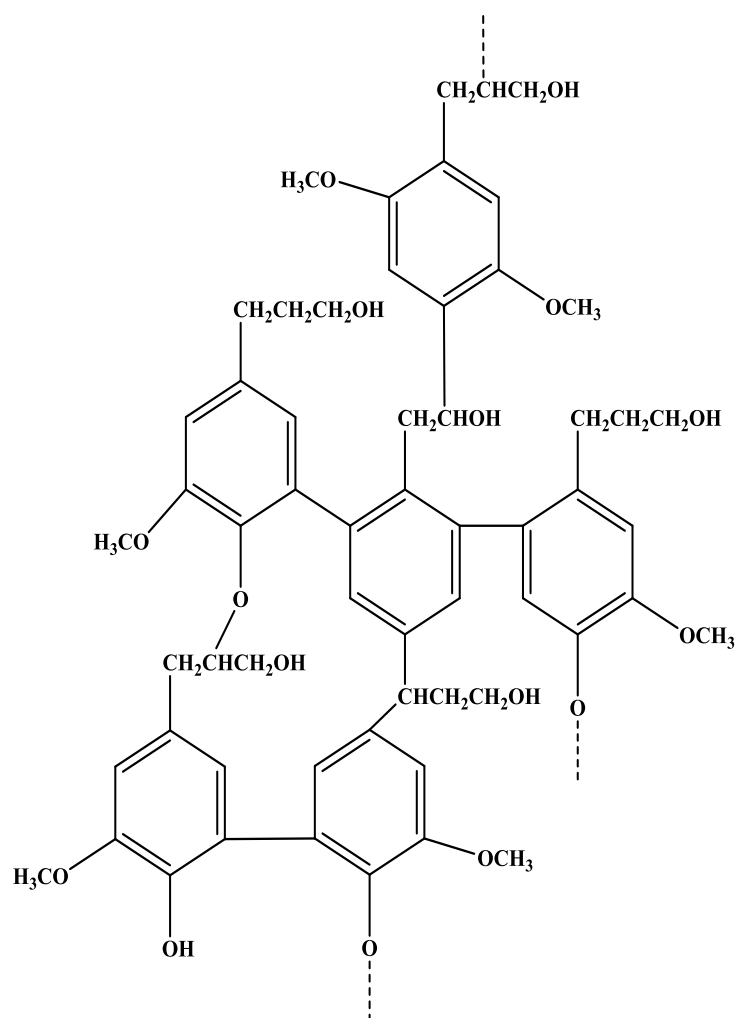


Figure 2.4. Hypothetical structure of lignin

2.3.4. Phenolic compounds

Phenolic compounds, among other important bioactive compounds of plants, represent a wide variety of compounds characterized by a phenolic structure (aromatic ring(s) bearing one or more hydroxyl groups) (Denny and Buttriss, 2007). Phenolic structures are diverse and can be classified into different groups (Scalbert *et al.*, 2005). Distinctions are thus made between phenolic acids, flavonoids, lignans, and stilbenes, among others. In addition to this diversity, phenolic compounds may be associated with carbohydrates (simple and complex), lipids, organic acids, and some can also be linked to cell wall components such as cellulose, hemicelluloses, and lignin (Bravo, 1998).

Phenolic compounds can be classified as soluble (SP) and insoluble-bound (IBP) phenolics. The soluble forms are simply extracted using organic solvents and occur in both the free and soluble ester forms (Antolovich *et al.*, 2000). Bound phenolic compounds are typically involved in cell wall structure and require acid or base hydrolysis to release them from the cell wall matrix. The level of phenolics in plant sources also depends on cultivation techniques, cultivar, growing conditions, extraction methods and extraction temperature, ripening process, as well as processing and storage conditions (Ongphimai *et al.*, 2013).

2.4. Phenolics and their classification

Phenolics and polyphenolics constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with over 8000 known phenolic structures currently published (Tsao, 2010). These compounds can be subdivided into different classes according to the number of their phenol rings and the structural elements linked to the basic units (Kim *et al.*,

2006). Table 2.1 shows a general classification of the 21 principal structures based on the number of carbons in the molecule (Figure 2.5).

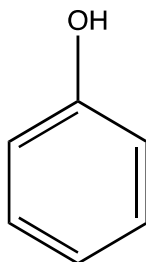


Figure 2.5. Basic structure of a phenol

The term phenolics cover a very large and diverse group of chemical compounds. These compounds can be classified in a number of ways. Harborne and Simmonds (1964) have classified these compounds into groups based on the number of carbons in the molecule as simple phenolics, phenolic acids and related compounds, such as acetophenones and phenylacetic acids, cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols, coumarins, isocoumarins, and chromones, chalcones, aurones, dihydrochalcones, flavans, flavones, flavanones, flavanonols, anthocyanidins, anthocyanins, biflavonyls, benzophenones, xanthonenes, stilbenes, quinones, lignans, neolignans (dimers or oligomers), lignin (polymers), tannins (oligomers or polymers), and phlobaphenes (polymers) (Table 2.1). Several types of plant materials, such as vegetables, fruits, seeds, hulls, wood, bark, roots and leaves, spices, herbs, etc. have been examined as potential sources of phenolic compounds with antioxidant activity (Moure *et al.*, 2000; Soong and Barlow, 2004; Rubilar *et al.*, 2006).

Table 2.1. Classification of phenolic compounds

Structure	Class
C ₆	Simple phenolics
C ₆ -C ₁	Phenolic acids and related compounds
C ₆ -C ₂	Acetophenones and phenylacetic acids
C ₆ -C ₃	Cinnammic acids, cinnamyl aldehydes, cinnamyl alcohols
C ₆ -C ₃ -C ₆	Coumarins, isocoumarins, and chromones
C ₁₅	Chalcones, dihydrochalcones
C ₁₅	flavans
C ₁₅	flavones
C ₁₅	flavanones
C ₁₅	flavanonols
C ₁₅	anthocyanidins
C ₁₅	anthocyanins
C ₆ -C ₁₀ -C ₁₄	quinones
C ₁₈	betacyanins
Lignans, neolignans	dimers or oligomers
Lignins	polymers
Tannins	oligomers or polymers
Phlobaphenes	polymers

2.4.1. Major phenolics in woody plants

2.4.1.1. Phenolic acids (hydroxybenzoic and hydroxycinnamic acids)

Phenolic acids, known to serve as multipurpose bioactive compounds, are widely spread throughout the plant kingdom. Most of them are an integral part of the human diet, and are also consumed as medicinal preparations. Many of the health protective effects of phenolic compounds have been ascribed to their antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antimicrobial, and other biological properties (Xu *et al.*, 2008). Substituted derivatives of hydroxybenzoic and hydroxycinnamic acids are the predominant phenolic acids in plants, with hydroxycinnamic acids being the more common. These derivatives differ in the pattern of the hydroxylation and methoxylation in their aromatic rings (Shahidi and Naczki,

2004a; Mattila and Hellström, 2007). Technically speaking, only benzoic acid derivatives are phenolic acids and cinnamic acid derivatives are phenylpropanoids. The basic pathway for synthesis of phenolic acids in plants begins from sugars through to aromatic amino acids – phenylalanine, and, in some rare cases, tyrosine. The formation of *trans*-cinnamic acid from phenylalanine and *p*-hydroxycinnamic acid from tyrosine is catalyzed by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively (Amarowicz *et al.*, 2009) (Figure 2.6).

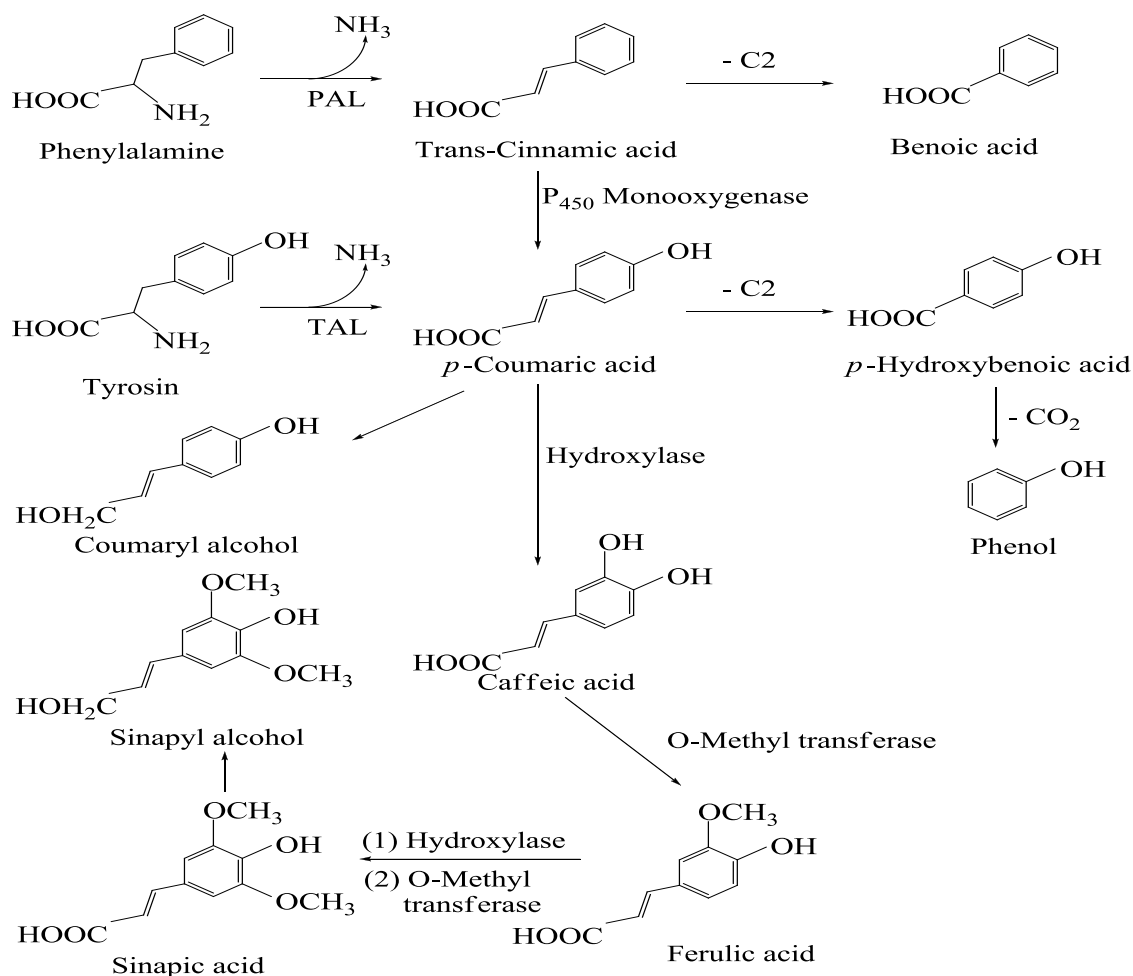
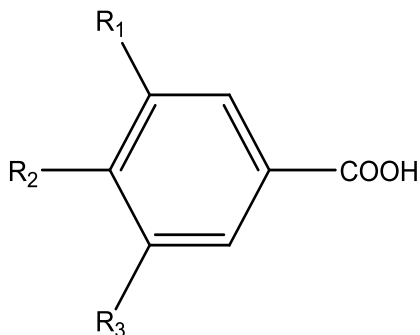


Figure 2.6. Formation of phenylpropanoids of cinnamic acid family as well as benzoic acid derivatives and corresponding alcohols from phenylalanine and tyrosine; PAL: phenylalanine ammonia lyase; and TAL: tyrosine ammonia lyase

Phenolic acids are present in some plant foods mostly in the bound form. The most common hydroxycinnamic acids are caffeic, *p*-coumaric and ferulic acids (Figure 2.7), which frequently occur in foods as simple esters with quinic acid or glucose. Probably the most well-known bound hydroxycinnamic acid is chlorogenic acid, which is an ester of caffeic and quinic acids. Unlike hydroxycinnamates, hydroxybenzoic acid derivatives are mainly present in foods in the form of glucosides; *p*-hydroxybenzoic, vanillic, and protocatechuic acids are the most common forms (Herrmann, 1989; Shahidi and Naczk, 2004a,b; Mattila and Hellström, 2007; Shahidi, 2009).

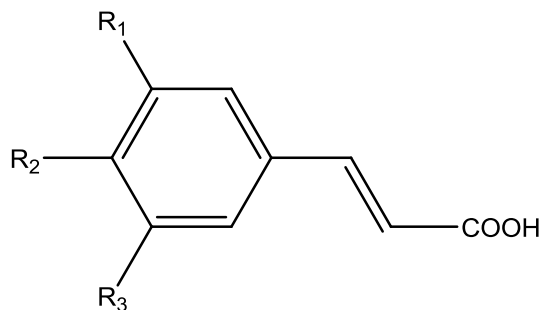
Phenolic acids behave as antioxidants, due to the reactivity of their phenol moiety (hydroxyl substituent on the aromatic ring). Although there are several mechanisms, the predominant mode of antioxidant activity is believed to be radical scavenging via hydrogen atom donation. Other established antioxidant, radical quenching mechanisms are through electron donation and singlet oxygen quenching (Shahidi and Wanasundara, 1992; Shahidi and Ambigaipalan, 2015). Substituents on the aromatic ring affect the stabilization and therefore the radical-quenching ability of these phenolic acids. Different acids therefore have different antioxidant activities (Rice-Evans *et al.*, 1996). The antioxidant behaviour of the free, esterified, and glycosylated phenolics have been reported (Robbins, 2003, Albishi *et al.*, 2013c).

There is an awareness and interest in the antioxidant behaviour and potential health benefits associated with these simple phenolic acids. It is their role as dietary antioxidants that has received the most attention in the literature (Rice-Evans *et al.*, 1996; Robbins, 2003). Because of their ubiquitous presence in plant-based foods, humans consume phenolic acids on a daily basis. The estimated range of consumption is 25 mg-1g per day depending on the diet consumed (fruit, vegetables, grains, teas, coffees, and spices) (Clifford, 1999).



Hydroxybenzoic acids

Acid	R ₁	R ₂	R ₃
<i>p</i> -Hydroxybenzoic	H	OH	H
Protocatechuic	OH	OH	H
Vanillic	OCH ₃	OH	H
Syringic	OCH ₃	OH	OCH ₃
Gallic	OH	OH	OH



Hydroxycinnamic acids

Acid	R ₁	R ₂	R ₃
<i>p</i> -Courmaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH ₃	OH	H
Sinapic	OCH ₃	OH	OCH ₃

Figure 2.7. Chemical structures of naturally occurring phenolic acids and related compounds

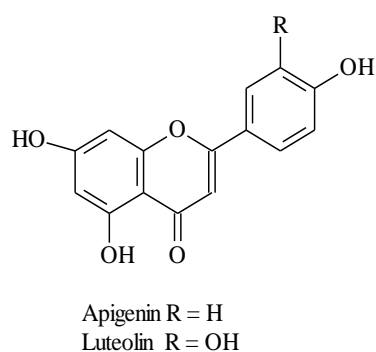
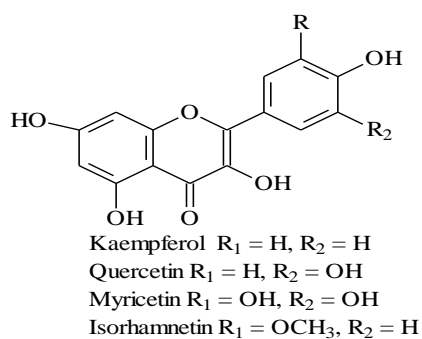
Phenolic compounds are commonly found in both edible and nonedible plants and have been reported to possess multiple biological effects, including antioxidant activity. Crude extracts of vegetables, herbs, fruits, cereals, and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. The importance of the antioxidant constituents

of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufactures, and consumers as the trend of the future is moving toward functional food with specific health effects (Kahkonen *et al.*, 1999). Polyphenols are recognized as the most abundant antioxidants in human diet (Manach *et al.*, 2004). As antioxidants, phenolic compounds prevent the formation of free radicals with deleterious health effects and are therefore important in disease risk reduction (Shahidi, 1992; 1997; 2000; Shahidi *et al.*, 1992). They have been demonstrated to have positive effects on certain types of cancer (Birt, 2006), including cancer of the stomachs, colon, prostate, and breast as well as cardiovascular disease (CVD) (Hertog *et al.*, 1995), and various inflammatory disorders (Andriantsitohaina *et al.*, 2012).

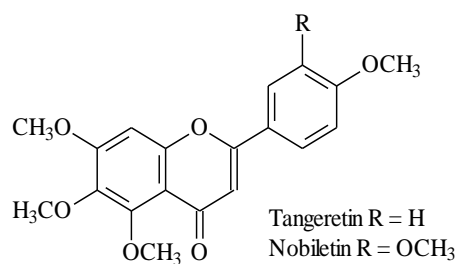
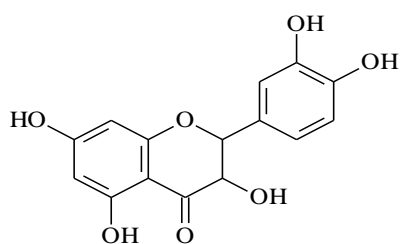
2.4.1.2. Flavonoids

Flavonoids contain a phenolic benzopyran structure (C6-C3-C6), where two aromatic rings are attached to a heterocyclic ring (Figure 2.8). As a function of the hydroxylation pattern of the ring heterocyclic, flavonoids are further classified into anthocyanin, flavan-3-ols, flavones, flavanones and flavonols (Tsao, 2010). The subgroup chalcones is also considered to be in the flavonoid family, even though it lacks the heterocyclic ring. Several thousand flavonoids have been discovered and there are probably many more to be identified (Harborne and Williams, 2000). In plants, flavonoids exist as either glycones or aglycones depending upon the glycosylation patterns. Like other phenolics, flavonoids are crucial for normal growth and development and defense systems in plants. Some flavonoids are responsible for imparting colour, flavour, odour to the flowers, fruits and leaves (Harborne, 1989; Gharras, 2009). Flavonoids constitute the largest subgroup of phenolics due to their presence as glycosides,

methoxides and various acylation patterns on the three rings (Figure 2.8). The examples are quercetin and kaempferol, which have 279 and 347 different glycosidic compounds, respectively (Tsao and McCallum, 2009; Williams, 2006).



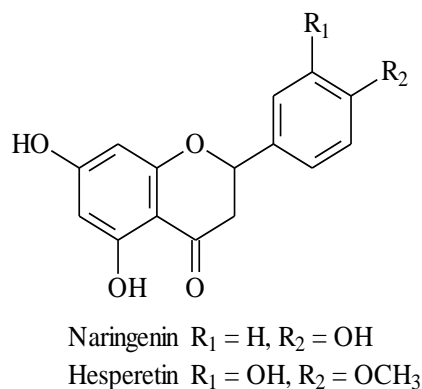
FLAVONOLS



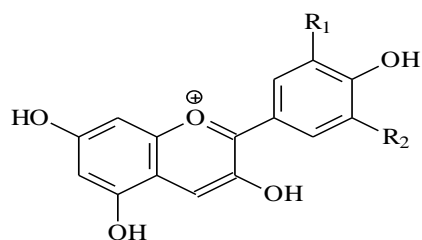
FLAVONES

Taxifolin

FLAVANONOLES



FLAVANONES



Anthocyanidin	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Pelargonidin	H	H
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OH	OCH_3

ANTHOCYANIDINS

Figure 2.8. Chemical structures of selected flavonoids

Human intake of all flavonoids is estimated at about a hundred milligrams, varying by nearly 50-fold, from 20 to 1,000 mg/day (Mullie *et al.*, 2007). The total average intake of flavonols (quercetin, myricetin, and kaempferol) was estimated at 23 mg/day, of which quercetin contributed ~70%, kaempferol 17%, and myricetin 6% (Hertog *et al.*, 1993).

2.4.1.3. Tannins

Tannins are polyphenolic compounds occurring in the barks, woods, and fruits of many kinds of plants. Extraction of tannins from the bark of different trees has been carried out (Balange and Benjakul, 2009; Fradinho *et al.*, 2002). Tannins comprise a group of compounds with a wide diversity in structure that shares their ability to bind and precipitate proteins. The name tannins refer to the process of tanning animal skin to form leather. This process has been known since prehistoric times, when animal hides were treated with animal fat and brain tissue. Chemically this resulted in the cross-linking of the collagen chains in the hide. Throughout much of history the tanning process was performed with tannins derived from plants, until minerals such as aluminum and chromium replaced the use of plant tannins during the last century. As part of Japanese and Chinese natural medicine, tannins have been used as anti-inflammatory and antiseptic compounds. They have also been used to treat a wide array of illnesses, including diarrhea and tumors in the stomach or duodenum (Khanbabaee and Van Ree, 2001). Tannins are abundant in many different plant species, particularly oak (*Quercus* spp.), chestnut (*Castanea* spp.), staghorn sumac (*Rhus typhina*), and fringe cups (*Tellima grandiflora*), among others. Tannins can be present in the leaves, bark, and fruits, and are thought to protect the plant against infection and herbivory (Khanbabaee and Van Ree, 2001). Tannins can be classified as condensed and hydrolysable tannins (Figure 2.9).

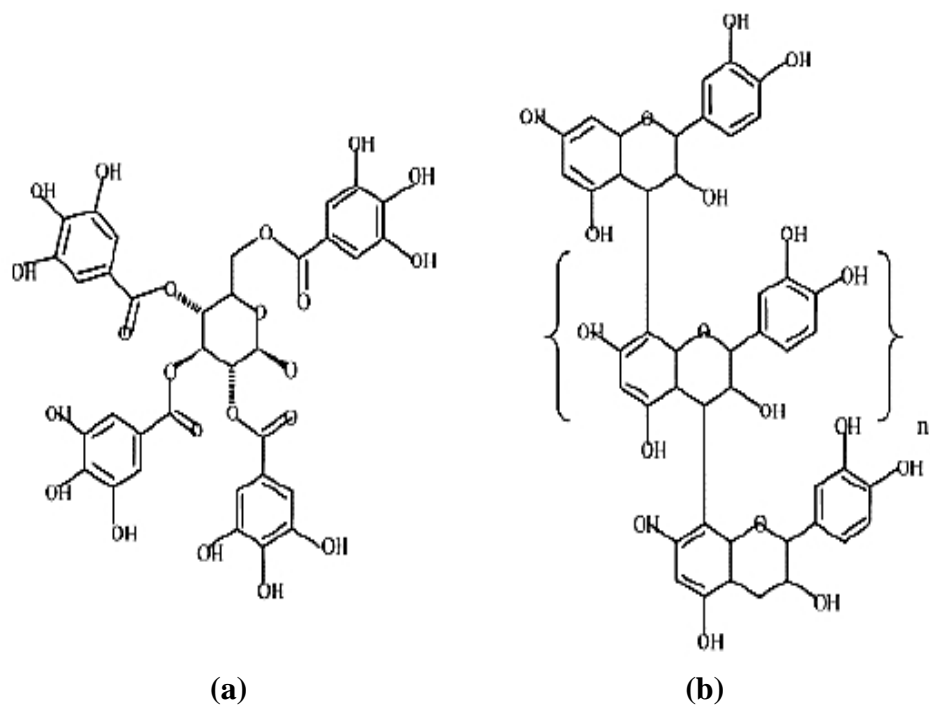


Figure 2.9. Diagrammatic representation of hydrolyzable (a) and condensed (b) tannins

2.4.1.4. Lignans

Lignans (Figure 2.10) are dimers or oligomers that result from the reaction of monolignol radicals which are derived from *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2.3), with coniferyl alcohol being the most common monolignol used in lignan biosynthesis. Lignans are localized in woody stems and in seeds and play a role as insect deterrents (Hatfield and Vermerris, 2001).

2.4.1.5. Lignin

Lignin is a phenolic polymer that belongs to polyphenol family. It is the second most abundant biopolymer on Earth (after cellulose), and plays an important role in providing structural support to plants. Its hydrophobicity also facilitates water transport through the vascular tissue. The chemical complexity and apparent lack of regularity in its structure make lignin extremely suitable as a physical barrier against insects and fungi (Vermerris and Nicholson, 2009). Like lignans, lignin is synthesized primarily from three monolignol precursors: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 2.3). Lignin is an important constituent of wood representing the 25-30% of its total weight. It is three-dimensional polymer, in which the monomer can be considered the phenyl propane, with one or more methoxy groups (-OCH₃) bonded to the aromatic ring (Figure 2.11).

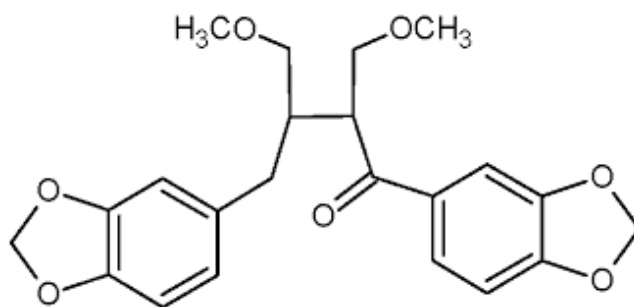


Figure 2.10. Basic chemical structure of lignan

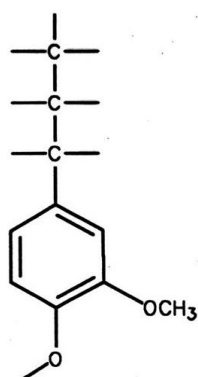


Figure 2.11. The phenylpropane unit

2.4.1.6. Soluble- and insoluble-bound phenolics

Phenolic compounds can be divided into free, esterified and insoluble-bound types, depending on whether they occur in a free or bound form. Soluble phenolics include free phenolics, which do not form a chemical bond with other molecules, and esters that are associated through ester bonds with fatty acids and glucuronic acid. These soluble phenolics are readily extracted by extraction medium, hence are also referred to as extractable phenolics. On the other hand, insoluble-bound phenolics are bound with macromolecules that are not extracted by extraction medium such as cellulose, hemicellulose, pectin, and structural protein. Thus, the chemical bond must first be broken via hydrolysis, chemically or enzymatically, to extract insoluble-bound phenolics from foods.

As mentioned earlier, most insoluble-bound phenolics are chemically and covalently bonded to cell wall components and these account for a relatively large proportion of the total phenolics (20–60% in vegetable, fruits, and legume/seeds) as compared to the soluble form in foods (Nayak *et al.*, 2015).

2.5. Phenolic antioxidant properties and their action mechanism

Most phenolic acids have antioxidant capacity, and radical scavenging ability (Cai *et al.*, 2006). The antioxidant potential of phenolic compounds depends on the number and arrangement of the hydroxyl groups in the molecules of interest (Cao *et al.*, 1997; Sang *et al.*, 2002). Phenolic antioxidants (AH) can donate hydrogen atoms to lipid radicals and produce lipid derivatives and antioxidant radicals (Reaction V), which are more stable and less readily available to promote autoxidation (Kiokias *et al.*, 2008). The antioxidant free radical may further interfere with the chain-propagation reactions (Reactions VI and VII).



As bond energy of hydrogen in a free radical scavenger decreases, the transfer of hydrogen to the free radical is more energetically favourable and thus more rapid (McClements and Decker, 2007). The phenoxyl radical is stabilized by delocalization of its unpaired electron around the aromatic ring (Figure 2.12) which participates in the termination reaction.

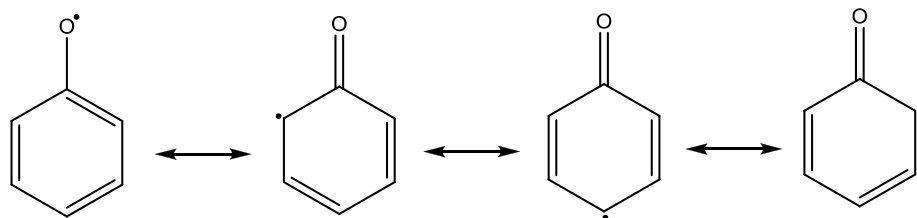
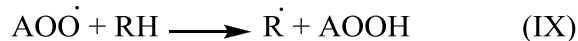
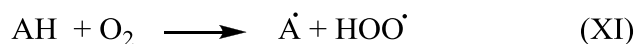


Figure 2.12. Resonance stabilization of phenoxyl radical

Gorden (1990) reported that substitution at the *para* position with an ethyl or n-butyl group rather than a methyl group improves the activity of the antioxidant; however, the presence of chain or branched alkyl groups in this position decreases the antioxidant activity. The stability of the phenoxyl radical is further increased by bulky groups in the 2 and 6 positions as in 2,6-di-*t*-butyl-4-methylphenol (BHT) (Miller and Quakenbush, 1957), since these substituents increase the steric hinderance in the region of the radical and thereby further reduce the rate of propagation reactions involving the antioxidant radical (Reactions VIII, IX, X).



The effect of antioxidant concentration on autoxidation rates depends on many factors, including the structure of the antioxidant, oxidation conditions, and the nature of the sample being oxidized (Shahidi and Naczki, 2004a). Often phenolic antioxidants lose their activity at high concentrations and behave as prooxidants (Gorden, 1990) by involvement in initiation reactions (Reactions XI, XII).



Phenolic antioxidants are more effective in extending the induction period when added to any oil that has not deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids (Mabarouk and Dugan, 1961). Thus, antioxidants should be added to foodstuffs as early as possible during processing and storage in order to achieve maximum protection against oxidation (Shahidi and Wanasundara, 1992).

Natural sources of antioxidants are mainly found in plant and non-plant sources. These include fruits, vegetables, their by-products, and wood. This section will explore some sources of natural antioxidants and will specifically discuss five types of woody plants as sources of natural antioxidants.

Naturally-occurring antioxidative compounds in plants include flavonoids, phenolic acids, lignans, terpenes, tocopherols, phospholipids and polyfunctional organic acids, among others. As

already mentioned, sources of natural antioxidants are primarily plant phenolics that occur in all parts of the plants. They can be found in fruits, vegetables, nuts, seeds, leaves, flours, roots and bark (Wanasundara *et al.*, 1996). There have been numerous studies on the biological activities of phenolics, which are potent antioxidants and free radical scavengers (Naczki and Shahidi, 2004; 2006; Tung *et al.*, 2007). Figure 2.12 provides the chemical structures of certain natural antioxidant compounds.

2.6. Natural antioxidants

Natural antioxidants have gained popularity due to the belief that natural food ingredients are better and safer than their synthetic counterparts. The natural antioxidants, primarily phenolics, occur in all parts of plants such as fruits, leaves, seeds, flowers, roots, and woods among others (Lee *et al.*, 2008). Other important natural antioxidants include carotenoids, and antioxidant vitamins (Gurib-Fakim, 2006). Most food companies would prefer natural antioxidants compared to synthetic antioxidants, and are interested in novel, natural sources that are effective and economical (Larrosa *et al.*, 2002). Therefore, it would be tremendously advantageous to identify them in wood. In the present study, wood and bark of some trees were investigated, with special attention to their phenolics and lignin components as natural antioxidants.

2.6.1. Date palm wood

Woody plants have recently attracted considerable research interest (Atawodi *et al.*, 2017). There is a significant number of date palm trees grown in Middle-East covering approximately 42% of the area. It may be noted that numerous extracts from various palm trees have many traditional uses without the active substances being actually known. Date palm tree species are very promising plant materials in the search for natural products with various biological activities because the plants grow under very severe conditions such as strong ultraviolet rays and high temperatures. Saudi Arabia is considered one of the pioneer countries in date palm cultivation and date production (Nasser, 2014). In Saudi Arabia, the date agricultural generates large amounts of dates that are usually used as nutritional sources. The annual production of dates is estimated at more than 970 thousand tons, produced from more than 23 million date palm trees,

spread throughout different regions of the country (Chandrasekaran and Bahkali, 2013). Each region is characterised by certain date palm cultivars. Although there are more than 400 date cultivars, approximately 60 cultivars are used commercially (Nasser, 2014). When a palm tree is growing, only 40% of the tree is used as a source of dates the rest, like bark which is most often not valued, is discarded (Al-Khalifah and Askari, 2003). Palm trees have been identified as a nutritional source of dates, which are abundant in phenolic compounds. The heartwood of some palm tree wood species have high extractive content, usually rich in polyphenols (Daayf *et al.*, 2003), which have many favourable effects on bioactivities such as inhibiting the oxidation of low-density lipoproteins (Frankel *et al.*, 1993), and also phenolics have anti-inflammatory properties (Albishi *et al.*, 2013b).

The date palm (*Phoenix dactylifera L.*) tree (Figure 2.13) is an important element of the flora in all Arab countries (Nasser, 2014) and plays a pivotal role in economic, social, and cultural life in Arab region. Date palm trees have resisted the harsh climatic and environmental conditions of the area and shown generous yields, while no other type of tree has been as successful under the same conditions (Nasser, 2014; Chandrasekaran and Bahkali, 2013). A massive quantity of biomass results annually from the seasonal pruning of the date palm population, which is an essential agricultural practice. In a study by El-Huhany (2001), an average of 35 kg of palm residues per tree was reported to be generated annually. Accordingly, the annual wasted date palm biomass in the form of residues from the seasonal trimming of the palm tree population in Saudi Arabia is estimated to be approximately one million metric tons. Most of these residues are burnt in developing countries, whereas in developed countries, they are used to produce wood composites, such as particleboard and medium density fiberboard.

Every part of the date palm tree can be utilized effectively, starting from the date fruit, including branches, bark, wood, and fibers. The residues of date palm trees have been used as a source of raw materials for the wood industry, for the production of pulp and paper (Khiari *et al.*, 2011), particleboard (Hegazy and Aref, 2010), wood-plastic composites (Aref *et al.*, 2013), and wood-cement composites (Nasser and Al-Mefarrej, 2011; Hegazy and Aref, 2010). It is important to investigate the phenolics and their antioxidant abilities of date palm wood as there are no previous studies in literature in this context.

2.6.2. Oak wood

Oak is a tree in the genus of the beech family (Figure 2.13), having approximately 600 species. Northern red oak is very common in north, east, and central states of Canada, and it is widely planted in the west, in Vancouver, Victoria, British Columbia (Mitchell, 1987). Oak belongs to the class of heartwood which contains a large variety of compounds, including polysaccharides, lignins, phenols, tannins, sugars, minerals, and many different flavouring substances (Vivas *et al.*, 2013). Oak wood has been found to be very resistant to insect and fungal attack because of its relatively high tannin content (Návojská *et al.*, 2012). It is also found to decrease oxidative stress and increase activity of antioxidant enzymes and total antioxidant capacity of plasma *in vivo* (Angeloni, 2014). Oak wood vinegar has been reported to render anti-inflammatory effects and helps draw insect toxins out of the body (Lee *et al.*, 2011).

2.6.3. Pinewood

The use of pine tree (Figure 2.13) for tanning has been known for a long time in the Middle East, Europe and North Africa (Nahal, 1962; Saad *et al.*, 2014). Pine knots are a potential source of

stilbenes, which has been used as bioactive and antimicrobial compounds in drugs and in food (Fang *et al.*, 2013). Natural antioxidants are found in high concentrations in pinewood and exhibit excellent antioxidant activity (Saha *et al.*, 2013). White pine (*P. strobus*) has been valued as source of medicinal principles and the extract from inner bark is also rich in tannins (Karonen *et al.*, 2004). The bark extracts from Scots pine (*P. sylvestris*) is a rich source of polyphenols including proanthocyanidins (condensed tannins), catechin derivatives, glycosylated stilbenoids and flavonoids (Pan and Lundgren, 1996). Pine roots were subjected to controlled extraction and the resulting material showed noticeable anticancer activity (Bradette and Hébert, 2008). The antioxidant activity of pine bark extracts has been highlighted for some time, particularly standardized French maritime pine bark extract (*P. pinaster*) which is marketed under different trade names (Rimbach and Virgili, 1999; Tourino *et al.*, 2005). This commercial extract is a mixture of flavonoids, mainly phenolic acids and procyanidins, and has a demonstrated capacity for scavenging free radicals generated by somatic cells under oxidative stress of various chemicals; those free radicals are known to be involved in degenerative diseases such as Alzheimer's, cardiovascular disease, arthritis and certain forms of cancer (Royer *et al.*, 2013). *P. pinaster* bark extract is often used as the benchmark when screening for antioxidant activities of new natural extracts. Stevanovic *et al.* (2009) have reported the chemical composition and antioxidant properties of hydrophilic extracts from wood knots. Jack pine knot extracts were shown to inhibit oxidative processes in human cells (Phelan *et al.*, 2009).

2.6.4. Quebracho wood

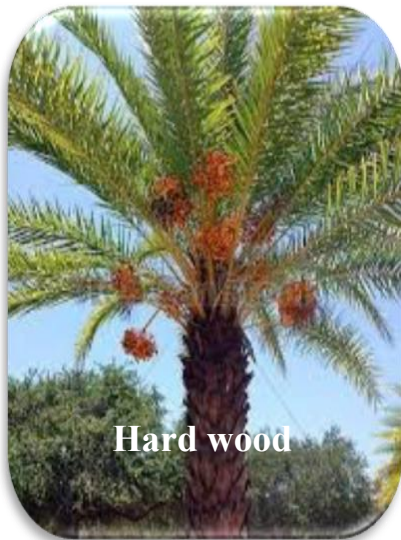
Quebracho (Figure 2.13) is a common name in Spanish to describe very hardwood tree species. Quebracho wood is red-coloured and very hard (Kirby and White, 1995). The two popular

species of quebracho tree, *Schinopsis balansae* and *Schinopsis lorentzii*, are grown in South America, particularly in north of Argentina and eastern Paraguay. These species are of interest, given the high concentration of phenolics that they accumulate. Extracts of quebracho phenolics have been tested to control gastrointestinal parasites in ruminants (Paolini *et al.*, 2003a,b; Athanasiadou *et al.*, 2001a,b, 2000). Quebracho phenolics have been classified as condensed tannins (Suschetet and de Larturieri, 1978; Athanasiadou *et al.*, 2001a; Lopez-Fluza *et al.*, 2003) and determined as gallic acid (Kratzer *et al.*, 1975); however, the separation and quantification of quebracho phenolics has not been carried out. The most common use of quebracho phenolics is in tannery processes (Marín-Martínez *et al.*, 2009). According to King and White (1957), the hydrolysable tannins and gallic acid found in the softwood constitute the raw material for the biosynthesis of the condensed tannins found in the hardwood. The tanning properties of quebracho extracts were discovered in 1867 by a French tanner, Emilio Poisier, who lived in Argentina. By 1895, the quebracho extracts were exported to Europe and became the principal vegetal tannin source in the world. The quebracho tannins structure like arabinol is very similar to that of grape tannins, making them a desirable alternative because they are less expensive to produce than grape tannins (Luz *et al.*, 2008).

2.6.5. Banana tree (Bark)

Banana plant (Figure 2.13), a monocotyledonous annual herbaceous plant, has been suggested as a suitable crop for several types of applications such as vegetable fibres, alternative to wood raw materials, for the pulp and paper applications and biocomposites (Cordeiro *et al.*, 2004, 2005; Faria *et al.*, 2006). Annually, about 72.5 million tons of bananas are produced (FAO, 2006), the *Cavendish* variety being the most produced and exported, corresponding to about 1/3 of the world production. The banana leaves often serve as a wrapping for grilling food. The leaves

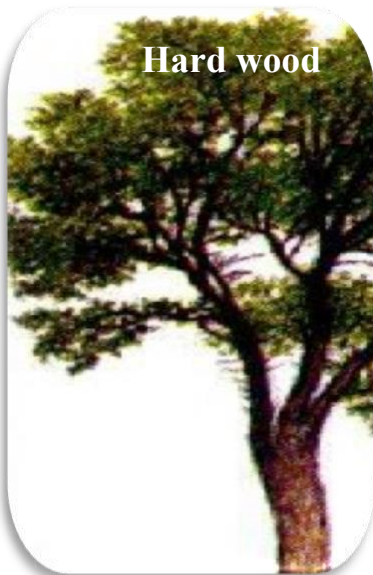
contain the juice that protects food from burning and adds a subtle flavour, while the banana fibre is used in the production of banana paper. Banana paper is made from two different parts: the bark of the banana plant which is mainly used for artistic purposes, or from the fibres of the stem and non-usable fruits (Gupta, 2014). Since such plant materials are available in the banana producing regions throughout the year, these can be an important industrial source of fibre and chemicals, thus constituting an additional economical profit to farmers (Reddy and Yang, 2005). The banana tree as an annual herbaceous plant that produces fruit all year around, could potentially serve as an inexpensive and readily available non-wood, renewable source of biomass. One of the macromolecular components that plays an important physiological role in the plant and determine the chemical processing of cellulosic fibres is banana lignin (Terashima and Fukushima, 1993).



(Phoenix dactylifera)
Date palm tree



(Quercus robur L.)
Oak Tree



(Schinopsis balansae)
Quibracho



(P.strobus)
Pine Tree



(Musa acuminata)
Banana (Bark)

Figure 2.13. Different varieties of wood

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

The following woods were used in the present investigation: Seedling date palm wood (SPW), Saudi old date palm wood (OPW), Oak wood (OW), Quibracho (QW), Pinewood (PW), Banana (BW). The heartwood from OPW was 28 years old, SPW tree 4 years old; OW and PW sapwood were collected in May-2014. Moisture content of the woods (an average of 3 trials; the quoted uncertainty is standard deviation) were found to be 22.8 ± 0.1 , 17.1 ± 0.2 , 18.5 ± 0.2 , 15.6 ± 0.1 , 10.01 ± 0.4 , and $13.5 \pm 0.23\%$ for SPW, OPW, OW, PW, QW and BW, respectively.

OPW and SPW woods were collected manually from the Salman Alfarsi Garden, Almadinah, Saudi Arabia, and the OW, PW, QW, and BW samples, were collected from the French Company Industrial de la Matiere Vegetale (CIMV). All the samples were freeze dried for 7 days at -48°C and 30×10^{-3} mbar (Freezone 6, model 77530, Labconco Co., Kansas City, MO). The dried samples were then ground, vacuum packed and stored in a freezer at -20°C until used for analysis within 5 days (Figure 3.1). All experiments were carried out in triplicate and the results were reported as mean \pm standard deviation. The methodologies followed are described below.

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was purchased from Acros Organics (Fair Lawn, NJ). Organic solvents and reagents such as methanol, acetone, n-butanol, and sodium carbonate were purchased from Fisher Scientific Co. (Nepean, ON). 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), Folin and Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and all phenolic compound standards with a purity of $\geq 96\%$ were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). Hydrogen peroxide, sodium hydroxide, 5,5-

dimethyl-1-pyrroline-*N*-oxide (DMPO), ferrous sulphate, ferric chloride as well as mono- and dibasic sodium and potassium phosphates, ethylenediaminetetraacetic acid (EDTA), deoxyribonucleic acid (DNA) of pBR 322 (*E.coli* strain RRI) and human LDL cholesterol were also purchased from Sigma-Aldrich Canada Ltd.



Figure 3.1. Wood samples (a) wood chips and (b) ground wood

3.2. Methods

3.2.1. Extraction of phenolics

Soluble and insoluble-bound phenolic compounds were extracted and fractionated as described by Krygier *et al.* (1982) and modified by Naczki and Shahidi (2006). Freeze dried woods (5g) were ultrasonicated for 20 min at 30°C with 150 mL of a mixture of methanol–acetone–water (7:7:6, v/v/v). The resulting slurries were centrifuged at 4000 x *g* (ICE Centra MS, International Equipment Co., Needham Heights, MA) for 5 min and the supernatants collected. The residue was re-extracted under the same conditions. After centrifugation, the combined extracts were analyzed for soluble phenolic acids, and the residue was reserved for determination of insoluble-bound phenolics. The combined supernatants were evaporated under vacuum at 40°C to remove

the organic solvents, and the aqueous phase was adjusted to pH 2 before extraction with hexane to remove interfering lipids (Krygier, 1982).

The soluble phenolic acids were then extracted 4 times with diethyl ether-ethyl acetate (1:1, v/v), dried under vacuum using a rotary evaporator and the extract was dissolved in 5 mL of 80% methanol. The residues were initially dispersed in 50 mL of 4 M NaOH and stirred for 4 hours under nitrogen. The solution was then acidified to pH 2, centrifuged and the bound phenolics were extracted with diethyl ether-ethyl acetate (1:1, v/v).

3.2.2. Determination of total phenolic content

The total phenolic content was determined according to an improved version of the procedure explained by Singleton and Rossi (1965). The Folin Ciocalteu's phenol reagent (1mL) was added to centrifuge tubes containing 1mL of methanolic extracts. Contents were mixed thoroughly and 1mL of sodium carbonate (75g/L) was added to each tube. To the mixture, 7mL of distilled water were added and mixed thoroughly. Tubes were then allowed to stand for 45min at ambient temperature. Contents were centrifuged for 5min at 4000xg (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the supernatant was read at 725nm. A blank sample for each extract was used for background subtraction. Content of total phenolics in each extract was determined using a standard curve prepared for gallic acid. Total extracted phenolics were expressed as mg of gallic acid equivalents per gram of extract.

3.2.3. Determination of total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen *et al.*, 1999). One mL of extracts or standard solution of quercetin (0.75, 1.5, 3 mg/mL)

was added to 10mL volumetric flask containing 4 mL distilled water. To the flask, 0.3 mL of 5% NaNO₂ was added. After 5 min, 0.3 mL 10% AlCl₃ was added. At the 6th min, 2 mL 1M NaOH solution were added and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/g dry plant material. Samples were analyzed in triplicate and the results were expressed as mean ± standard deviation.

3.2.4. Determination of condensed tannins

Determination of condensed tannins by the vanillin assay was based on the procedure reported by Scalbert *et al.* (1999). One mL of aqueous extract, contained in a test tube, was mixed with 2 mL of freshly prepared 1 % vanillin–sulfuric acid (70 %). The mixture was allowed to stand for 15 min at 20°C in a water bath. The absorbance of the mixture was measured at 500 nm. A catechin aqueous solution (30 mg/L) was used for calibration. The final results were expressed as mg catechin equivalent (CE) per g of dry weight (DW). Cyanidin equivalent content about 0.5 mL of aqueous extract was added to 5 mL of an acid solution of ferrous sulfate [77 mg of FeSO₄.7H₂O in 500 mL of 2:3 (1.18 M HCl/ n-BuOH)]. The tubes were loosely covered and placed in a water bath at 95°C for 15 min. The absorbance was read at 530 nm, and results were expressed as cyanidin gram equivalent (Cya) per g of dry weight (DW) (Scalbert *et al.* 1999). The condensed tannin content was calculated using the formula given below:

$$[CT] = \frac{A \times V \times D_f \times V' \times M}{\epsilon \times v \times m}$$

Where [CT] is the condensed tannin content (mg Cya/g DW); A is the absorbance of the sample; V is the volume of the reaction medium; V¹ is the volume of the aqueous extract recovered after

extraction with diethyl ether; v is 0.5 mL; D_f is the dilution factor; M is the molecular weight of cyanidin; m is the weight of the dry matter; and ϵ is the molar extinction coefficient ($34,700 \text{ M}^{-1} \text{ cm}^{-1}$).

3.2.5. Determination of hydrolyzable tannin content

Hydrolyzable tannins were determined by the method of Bossu *et al.* (2006) with slight modifications (Ben Mahmoud *et al.*, 2015). Five millilitres of KIO_3 aqueous solution (2.5%) were heated for 7 min at 30°C , and, then 1 mL of the sample was added. After additional 2 min of tempering at 30°C , the absorbance was measured at 550 nm. A calibration curve was obtained using tannic acid solution (5,000 mg/L) prepared by solubilization of 0.25 g of tannic acid in 50 mL of methanol (80%). The analytical standard solutions of tannic acid were prepared by aqueous dilution. Results were expressed as mg tannic acid equivalents (TAE) per g of wood extract. The experiment was carried out in triplicate.

3.2.6. Determination of lignin content from extracted woods

Lignin content from extracted woods was determined by the method of Moreira-Vilar *et al.* (2014) with slight modifications. Two millilitres of each sample was placed into a screwcap centrifuge tube containing 0.5 mL of 25% acetyl bromide and incubated at 70°C for 30 min. After complete incubation, the samples were quickly cooled in an ice bath, and then mixed with 0.9 mL of 2 M NaOH, 0.1 mL of 5 M HCl, and the samples were centrifuged. After centrifugation (1,400g, 5 min), the absorbance of the solution was measured at 280 nm. A standard curve was generated with alkali lignin (obtained from Sigma Aldrich) and the results were expressed as mg/ g lignin of dried wood sample.

3.2.7. Determination of the effect the antioxidant activities of soluble and insoluble-bound phenolics extracted from wood varieties

3.2.7.1. Measurement of total antioxidant capacity by trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on scavenging of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical anion (ABTS^{•+}). A solution of ABTS^{•+} was prepared in 2.5mM saline phosphate buffer (PH 7.4, 0.15M sodium chloride) (PBS) by mixing 2.5mM 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.0 mM ABTS^{•+}. The solution was heated for 16 min at 60°C, protected from light and stored in the dark at room temperature until used. The radical solution was used within 2 h as the absorbance of the radical itself decreases with time. Wood extracts were dissolved in PBS at a concentration of 0.17mg/ mL and diluted accordingly to have them fit in the range of values in the standard curve. For measuring antioxidant capacity, 40 µL of the sample were mixed with 1.96 mL of ABTS^{•+} solution. Absorbance of the above mixture was monitored at 734 nm over a six min. period. The decrease in absorbance at 734 nm, 6 min. after the addition of a test compound, was used for calculating TEAC values. A standard curve was prepared by measuring the reduction in absorbance of ABTS^{•+} solution at different concentrations of trolox. Appropriate blank measurements (decrease in absorption at 734 nm due to solvent without any compound added) were made and the values recorded (Van den Berg *et al.*, 1999) as modified by Siriwardhana and Shahidi (2002).

TEAC values were determined as follows:

$$\Delta A_{\text{trolox}} = \{A_{t=0 \text{ trolox}} - A_{t=6 \text{ min trolox}}\} - \Delta A_{\text{solvent (0-6 min)}}$$

$$\Delta A_{\text{trolox}} = m \times [\text{trolox}]$$

$$\text{TEAC} = \{\Delta A_{\text{extract}} / m\} \times d$$

Where, ΔA = reduction in absorbance, A = absorbance at a given time, m = slope of the standard curve, $[\text{trolox}]$ = concentration of trolox, and d = dilution factor.

3.2.7.2. DPPH radical scavenging capacity (DRSC) using electron paramagnetic resonance (EPR)

DRSC assay was carried out using the method explained by Madhujith and Shahidi (2006). Two millilitres of 0.18 mM solution of DPPH in methanol were added to 500 μ L of appropriately diluted soluble and bound phenolics extracts in methanol. Contents were mixed well, and after 10min. the mixture was passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billerica, MA). The spectrum was recorded on Bruker E-scan food analyzer (Bruker Biospin Co.). The parameters were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, 1.86 G modulation amplitude. For quantitative measurements of radical concentration remaining after reaction with the extracts, the method of comparative determination based on the corresponding signal intensity of first-order derivative of absorption curve was used. *DRSC* of the extracts was calculated using the following equation:

DPPH radical scavenging capacity % =

$100 - (\text{EPR signal intensity for the medium containing the additive} / \text{EPR signal intensity for the control medium}) \times 100$.

From the standard curve plotted for the DRSC of trolox, the scavenging activity of wood extracts was determined and expressed as $\mu\text{mol TE /g extract wood}$.

3.2.7.3. Reducing power activity

The reducing power of wood extracts was determined by the method of Albishi *et al.* (2013c). Briefly, each extract (0.2–1.0 mg) was dissolved in 1.0 mL of distilled water to which was added 2.5 mL of a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of a 1% (w/v) solution of potassium ferricyanide for determination of reducing power. The mixture was incubated in a water bath at 50 °C for 20 min. Subsequently, 2.5 mL of a 10% (w/v) solution of trichloroacetic acid were added and the mixture was subsequently centrifuged at 1750×g for 10 min. afterwards, a 2.5-ml of the supernatant was combined with 2.5 mL of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride. Absorbance of the reaction mixture was read spectrophotometrically at 700 nm; the increased absorbance of the reaction mixture indicates greater reducing power. Results were expressed as μ moles trolox equivalents of the extract versus absorbance at 700 nm.

3.2.7.4. Measurement of iron (II) chelation capacity

The chelation of ferrous ions by extract was estimated by the method of Liyana-Pathirana *et al.* (2006) with some modifications. In brief, 0.5 mL of extract was mixed with 1.85 mL of methanol and 0.05 mL of 1 mmol/L ferrozine, followed by vigorous shaking and allowing the mixture to react at room temperature for 10 min. The absorbance was measured spectrophotometrically at 562 nm. The chelation capacities of woods were expressed as μ mol ethylenediaminetetraacetic acid (EDTA) equivalents/g extract. The blank was devoid of ferrozine. Iron chelation capacities of the extracts were calculated using the following equation:

Fe (II) chelation capacity, % = $(1 - \text{Absorbance}) \times 100 / \text{blank Absorbance}$

3.2.8. *In Vitro biological activities of wood extracts*

While the in vitro biological activities of wood extracts have been assist in this work, in vivo studies have not been included. Thus, absorbtion and metabolism of bioactive compounds present has not been assessed and considering beyond the scope of this research.

3.2.8.1. *Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation*

The inhibitory effect of wood extracts on cupric ion-induced human LDL peroxidation was determined according to the method described by Ambigaipalan and Shahidi (2015). Initially, 5 mg/mL LDL was dialyzed against 100 volumes of freshly prepared 10 mM phosphate buffer (pH 7.4, 0.15 M NaCl). A dialysis tube with a molecular weight cut off of 12–14 kDa (Fischer, Carle and Kammerer Scientific, Nepean, ON) was used to dialyze at 4 °C under a nitrogen blanket in the dark for 12 h. Diluted LDL cholesterol (0.04 mg LDL/mL) was mixed with the wood extracts dissolved in phosphate buffer (0.1 mg/mL). The samples were pre-incubated at 37 °C for 15 min, and the reaction was initiated by adding a solution of cupric sulfate (0.1 mL, 100 μ M). Then the samples were incubated at 37 °C for 22 h. The formation of conjugated dienes (CD) was recorded at 234 nm using a diode array spectrophotometer (Agilent, Palo Alto, CA). Tannic acid (100 ppm) was used as a positive control. The appropriate blanks were run for each sample by replacing LDL cholesterol and CuSO₄ with distilled water for background correction.

3.2.8.2. *Supercoiled strand DNA scission by peroxy and hydroxyl radicals*

The inhibition activity of the wood extracts against supercoiled DNA strand scission induced by peroxy radical was evaluated according to the method of Albishi *et al.* (2013a). Plasmid supercoiled DNA (pBR 322) was dissolved in 10 mM phosphate buffered saline (PBS) pH 7.4. The DNA (4 μ L) was added to 2 μ L of extract samples, 4 μ L of AAPH (22.5 mM) dissolved in

PBS. For peroxy radical-induced oxidation, the mixture was mixed well and incubated at 37°C for 1h. Upon completion of incubation, 2 µL of the loading dye (consisting of 0.25% bromophenol blue and 0.25% xylene cyanol) was added to the extracts and loaded to a 0.7% (w/v) agarose gel. The gel was prepared in 40 mM Tris-acetic acid- 1 mM EDTA buffer, pH 8.5. Thereafter, 5 µL SYBR Safe were added to DNA gel, and the samples were electrophoresed at 85 Volt for 75 min at 4 °C. DNA strands were visualized under ultraviolet light. For hydroxyl radical-induced DNA oxidation, 2 µL of test compounds, dissolved in methanol, were added into an Eppendorf tube and the solvent was evaporated under a stream of nitrogen. To the tube, 2 µL of distilled water were added, followed by thorough vortexing for 1 min. The following reagents were then added to the tube in the order stated: 2 µL of PBS (pH 7.4), 2 µL of supercoiled pBR322 DNA, 2 µL of H₂O₂ and 2 µL of FeSO₄. The mixture (10 µl) containing 1 µM test compound, 0.1 M PBS, 10 µg/mL DNA, 0.2 mM H₂O₂ and 0.1 mM FeSO₄ (final concentration/assay) was incubated at 37°C for 1 hour.

The protective effect of extracts was calculated as DNA retention (%) based on the following equation:

$$\text{DNA retention (\%)} = (\text{Supercoiled DNA content in sample} / \text{Supercoiled DNA in control}) * 100$$

3.2.8.3. *α-Glucosidase inhibitory activity*

The *α*-glucosidase inhibitory activity of wood extracts was measured according to the method of Eom *et al.* (2012) with slight modifications. For this assay, extracted samples in methanol were used without any dilution. Each wood test sample dissolved in methanol (10 µL) was mixed with 620 µL of potassium phosphate buffer (0.1 M, pH 6.8) in an Eppendorf tube. *α*-Glucosidase (10 U/ mL, 5µL) dissolved in potassium phosphate buffer (0.1 M, pH 6.8) was added to the sample

solution. After incubation at 37 °C for 20 min, substrate p-nitrophenyl glucopyranoside dissolved in distilled water (10 mM, 10 µL) was added to initiate the reaction. The reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 650 µL of 1 M Na₂CO₃. The amount of released product *p*-nitrophenol (yellow colour) was measured at 410 nm using a UV visible spectrophotometer. Sample blanks without enzyme and a control without sample were also measured. α-Glucosidase inhibition percentage was calculated using the equation:

α-Glucosidase Inhibitory Activity (%)

$$= \left(1 - \frac{\text{Absorbance of Sample} - \text{Absorbance of sample blank}}{\text{Absorbance of Control} - \text{Absorbance of control blank}} \right) \times 100$$

3.3. Analysis of phenolics and lignin extracted from woods

Many plants look similar to one another, especially wood parts of the trees. However, their composition of lignin (including phenolics) is different. Thus, identifying the lignin and phenolic compounds should be done by analysis determination to quantify and qualify the structure for each compound. To obtain more detailed information on the chemical structures of wood extracts, MALDI-TOF-MS, was performed.

3.3.1. MALDI-TOF-MS analysis of structural the phenolics and lignins of wood extracts

The extracts of SPW, OPW, OW, QW, PW, and BW were included in this study to demonstrate the clear advantages of MALDI-TOF-MS in revealing possible structural differences of phenolic acids, flavonoids, and lignins from different wood species. The MALDI-TOF-MS spectra were recorded on a Bruker Reflex III instrument (Bremen, Germany). The irradiation source was a

pulsed nitrogen laser with a wavelength of 337 nm, and the duration of the laser pulse was 3 ns. In the negative reflection mode, an accelerating voltage of 20.0 kV and a reflection voltage of 23.0 kV were used. The spectra of phenolics and lignin were obtained from a sum of 100-150 shots. 2,5- Dihydroxybenzoic acid (DHB, 1 mg/mL) was used as the matrix. The sample solutions (1 mg/mL) were mixed with the matrix solution at a volumetric ratio of 1:3. The mixture (1 µL) was applied to the steel target. Amberlite IRP-64 cation-exchange resin, equilibrated in deionized water, was used to deionize the analyte/matrix solution thrice.

3.4. Date palm as a new source of phenolic antioxidants and in preparing smoked salmon

The smoking process was carried out at the Marine Institute, St. John's, NL, Canada. Date palm wood was collected from Salman Al-Farsi farm, located in Madinah, Saudi Arabia, while maple (commercial) wood was kindly provided by the Marine Institute, Memorial University, St. John's, NL, Canada. Fresh fillets of salmon fish were purchased from a local grocery store in St. John's, NL, Canada. Raw fish was fresh as bought from the market (Figure 3.2). All the chemicals used in this study were of analytical grade and obtained from Sigma Aldrich. The vacuum bag (polyamide – PA), supplied by the PMT Company; Iceland was used in this study.



Figure 3.2. All materials that used in smoking process

3.4.1. Smoking process

The smoking process consists of placed fish fillets horizontally over smoke generated from wood smoldering inside a smokehouse, and the smoking has been duplicated by both woods date palm (SPW) and maple (MAW) as shown in Figure 3.3.

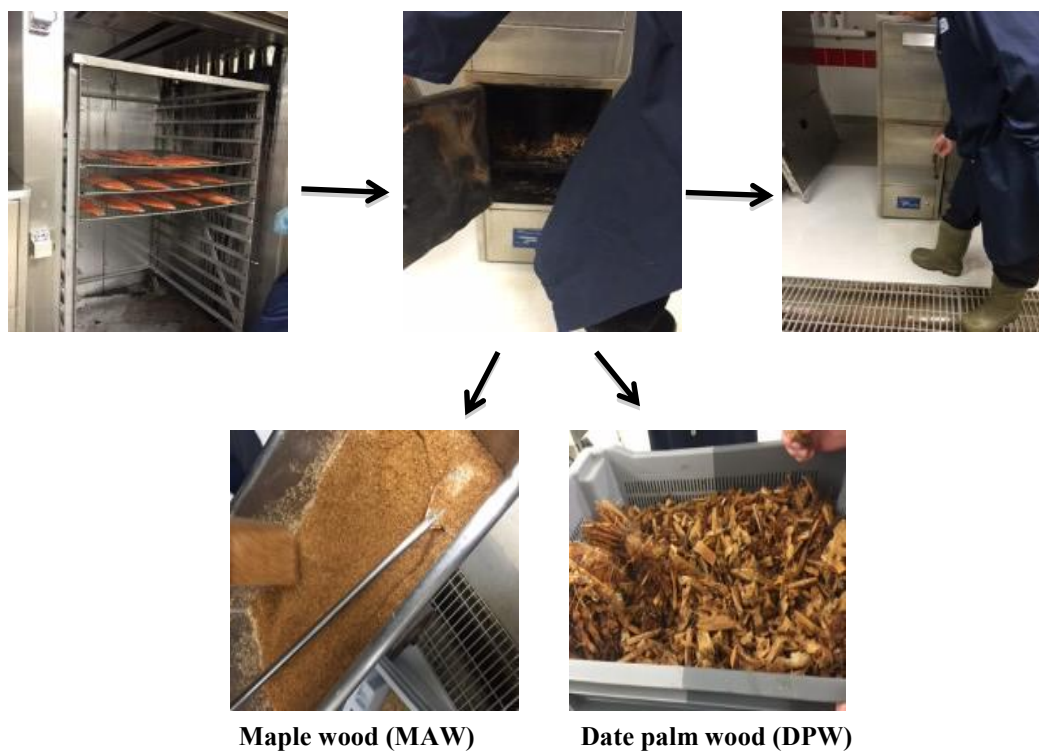


Figure 3.3. Smoking process by date palm and maple wood for salmon fillet

3.4.2 Extraction of soluble (SP) and insoluble-bound (IBP) phenolics from wood

Soluble and insoluble-bound phenolic compounds (crude extracts) were extracted as described earlier in this chapter ([Section 3.2.1](#)) and the extraction process has been presented briefly in Figure 3.4.

3.4.3. Sampling

Samples were taken on the arrival of the raw material, after smoking and after 1, 2, and 3 weeks of storage for evaluation of colour, thiobarbituric acid reactive substances (TBARS). While date palm wood (SPW) and maple wood (MAW) have been evaluated for their phenolic and

flavonoids contents, antioxidant activity using DPPH radical. GC-MS analysis was used for identifying the volatile phenolic produced during smoke processing.

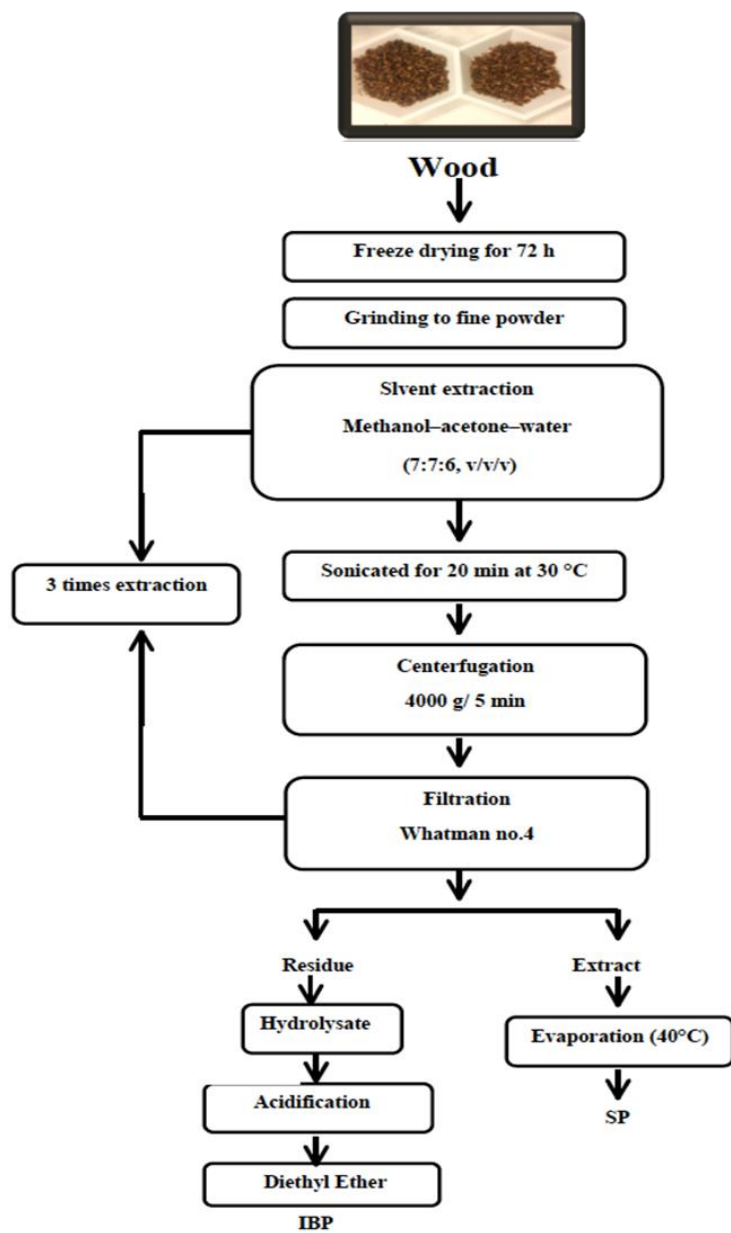


Figure 3.4. Extraction of SP & IBP phenolics from woods

3.4.4. Total phenolics (TPC) and flavonoids (TFC) contents of date palm wood and maple wood

The TPC (Singleton and Rossi, 1965) was determined according to an improved version of the procedure (Albishi et al., 2013b). The Folin Ciocalteu's phenol reagent (1 mL) was added to centrifuge tubes containing 1 mL of phenolic extracts. Contents were mixed thoroughly and 1 mL of sodium carbonate (75 g/L) was added to each tube. To the mixture, 7 mL of distilled water were added and mixed thoroughly. Tubes were then allowed to stand for 45 min at room temperature (23–25 °C). Contents were centrifuged for 5 min at 4000 x g (ICE Centra M5, International Equipment Co., Needham Heights, MA). The absorbance of the supernatant was read at 725 nm using an Agilent UV–visible spectrophotometer (Agilent 8453, Palo Alto, CA). The results were expressed as mg of gallic acid equivalents per gram of extract.

The TFC was measured by the aluminum chloride chlorimetric assay (Zhishen *et al.*, 1999). 1mL of extracts or standard solution of quercetin (0.75, 1.5, 3 mg/mL) was added to 10mL volumetric flask containing 4 mL distilled water. To the flask, 0.3 mL of 5% NaNO₂ was added. After 5 min, 0.3 mL 10% AlCl₃ was added. At the 6th min, 2 mL 1M NaOH solution were added and the total volume was made up to 10 mL with distilled water. The solution was mixed thoroughly and the absorbance was read against the prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/g dry plant material. Samples were analyzed in triplicates and the results were expressed as mean ± standard deviation.

3.4.5. DPPH radical scavenging capacity (DRSC)

DRSC assay was carried out using the method described by Madhujith and Shahidi (2006). Two millilitres of 0.18 mM solution of DPPH in methanol were added to 500 µL of phenolic extracts.

After 10 min, the mixture was passed through the capillary tubing, which guides the sample through the sample cavity of a Bruker e-scan EPR spectrophotometer (Bruker E-scan, Bruker Biospin Co., Billerica, MA). The spectrum was recorded on Bruker E-scan food analyzer (Bruker Biospin Co.). The parameters were set as follows: 5.02×10^2 receiver gain, 1.86 G modulation amplitude, 2.621 s sweep time, 8 scans, 100.000 G sweep width, 3495.258 G centre field, 5.12 ms time constant, 9.795 GHz microwave frequency, 86.00 kHz modulation frequency, 1.86 G modulation amplitude. For quantitative measurements of radical concentration remaining after reaction with the extracts, the method of comparative determination based on the corresponding signal intensity of first-order derivative of absorption curve was used. *DRSC* of the extracts was calculated using the following equation:

DPPH radical scavenging capacity % =

$100 - (\text{EPR signal intensity for the medium containing the additive} / \text{EPR signal intensity for the control medium}) \times 100$.

From the standard curve plotted for the *DRSC* of trolox, the scavenging activity of phenolic extracts was determined and expressed as $\mu\text{mol TE/g}$ of extract.

3.4.6. Determination of volatile compounds of smoking wood using solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC-MS)

The same fibre and minor modifications in the GC-MS method described by de Camargo *et al.* (2016) were used. The SPME was performed with a divinylbenzene (DVB)-Carboxen-polydimethylsiloxane (PDMS), 50/30 μm , 2 cm long fibre assembly coupled to a SPME manual holder assembly (Supelco, Bellefonte, PA). SPME fibre was preconditioned as recommended by the producer (1 h at 270 °C). The fibre was exposed to the smoke for 10 min, retracted into the

needle and injected into the gas chromatograph. GC-MS analyses of the volatile compounds adsorbed onto the SPME fibre were performed on a gas chromatograph GC 6890N (Agilent, Palo Alto, CA) coupled to a mass spectrometer 5973 (Agilent). A capillary column (DB-5MS 30 m \times 0.25 mm \times 0.25 μ m, Agilent) was used. The temperature program started at 40 °C. The temperature was raised to 60 °C at a rate of 5 °C/min after 3 min of holding time. This temperature was kept for 3 min, and then was raised to 200 °C at 8 °C/min, remaining at 200 °C for 10 min. Finally, the temperature was raised to 280 °C at 20 °C/min; remaining at this temperature for 5 min. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector (splitless mode) and the ion source were operated at 270 and 200 °C, respectively. Data integration was performed using the Chem Station software (Agilent). Volatile compounds were tentatively identified with the Wiley 9th Edition/NIST 2008 MS library and Kovats index, according to literature data (Adams, 1995; Dallüge *et al.*, 2002; Dötterl, Wolfe and Jürgens, 2005; El-Sayed *et al.*, 2005; Högnadóttir and Rouseff, 2003).

3.4.7. 2-Thiobarbituric acid reactive substances (TBARS) assay

Fish model systems were prepared as described previously (Albishi *et al.*, 2013b). Ground smoked fish (80g) was mixed with deionised water (20 mL) in Mason jars. Smoked fish samples were homogenised for 30s, transferred into plastic bags, and then stored in a refrigerator at 4 °C for 21 days. Samples for the analyses of TBARS were drawn on days 0 (fresh samples) and 21 days (3 weeks). TBARS were also determined using the method describe in the literature (Albishi *et al.*, 2013b). Two grams of each sample were weighed in a centrifuge tube to which 5mL of a 10% (w/v) solution of TCA was added and vortexed (Fisher Vortex Genie 2; Fisher Scientific, Nepean, ON) at high speed for 2 min. An aqueous solution (0.02 M) of TBA (5 mL)

was then added to each centrifuge tube, followed by further vortexing for 30s. The samples were subsequently centrifuged at 3000 *g* for 10 min and the supernatants were filtered through a Whatman No.3 filter paper. Filtrates were heated in a boiling water bath for 45 min, cooled to room temperature in cold water, and the absorbance of the resultant pink coloured chromogen read at 532 nm using an Agilent diode array spectrophotometer (Agilent 8453, Palo Alto, CA, USA). A standard curve was prepared using 1,1,3,3-tetramethoxypropane as a precursor of the malondialdehyde (MDA). The TBARS values were then calculated using the standard curve and expressed as milligrams MDA equivalents per kg of sample.

3.4.8. Colour measurement of smoked salmon

Colour parameters were determined by using a Hunter lab mini scan XE using the D65 light source (HunterLab, Reston, VA). The instrument recorded the *L* value, lightness on the scale of 0 to 100 from black to white; *a* value, (+) red or (-) green; *b* value, (+) yellow or (-) blue. The colour was measured above the lateral line at three positions: from the outside (A), middle (B), and skin (C) parts of each smoked fillet (Figure 3.5). Results are shown as the mean value of six measurements per fish fillet.

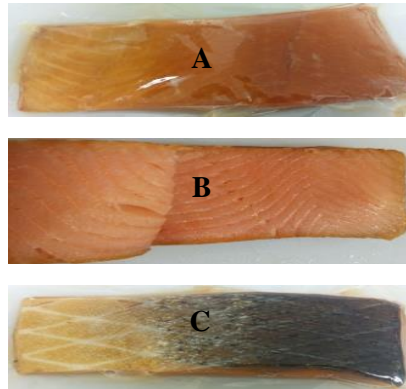


Figure 3.5. The colour measurement sampling spots: A (outside), B (middle), and C (skin) on the fillet

3.5. Statistical analysis

All experiments were carried out in triplicate and results were reported as mean \pm deviation. The significance of differences among the values was determined at $p < 0.05$ using analysis of variance (ANOVA) followed by Tukey's multiple range tests (Snedecor and Cochran, 1980).

CHAPTER 4

LOW MOLECULAR WEIGHT PHENOLICS OF DIFFERENT WOODS AND THEIR ANTIOXIDANT ACTIVITIES

4.1. Introduction

Interest in naturally occurring antioxidants has attracted increasing attention because of their environmental compatibility and safety for the consumers. The antioxidant activities of food polyphenols and processing by-products of the food industry are also interesting due to economic and ecological reasons (Moure *et al.* 2001; Yang *et al.* 2002; Louli *et al.* 2004). However, the use of by-products from mechanical and chemical processing of wood is poorly developed, although they have a high potential for the production of valuable products (Tao *et al.* 2002; Lapierre *et al.* 2004; Domenek *et al.*, 2013). Although antioxidant activities of low molecular weight plant polyphenols have been investigated for many years, their quantitative data were not well evaluated and compared with one another. At present, phenolic acids, flavonoids, lignans, and lignins are the best characterized antioxidants. The variety of polyphenols is large, and the diversity is further increased due to changes occurring during the course of isolation, particularly on the industrial scale (Crestini *et al.* 2011). In particular, woods (as by-products) would be very attractive for production of antioxidants particularly from an economic point of view (Louaifi *et al.* 2011; Domenek *et al.* 2013). The aim of the present work was to determine (identify?) phenolics isolated from various kinds of woods by means of solvent extraction using a variety of methanol-acetone-water procedures. The extracted antioxidants have been evaluated using DPPH[•] and ABTS^{•+}, which react with phenols by different mechanisms (Trouillas *et al.* 2008; Ponomarenko *et al.* 2014). Wood extracted low molecular weight compounds, mainly soluble

and insoluble-bound phenolics were also included in this study to recognize the tested woods as natural sources of antioxidants.

The extract yields reported in this study showed that seedling date palm wood (SPW) extract was highest compared to other wood extracts, particularly the Banana wood (BW) extract. The presence of concentrated active principles in the extracts was due to its prior extraction with a mixture of polar and non-polar solvents, which aided in the removal of interfering substances. Phenolics, flavonoids, and tannins are the principle compounds accounting for antioxidant potential and multiple biological effects. The total phenolic contents (TPC) of wood extracts observed in the present study is higher than those reported in certain previous studies (Singh *et al.*, 2007)

4.2. Determination of total phenolic content

4.2.1. Total phenolic content (TPC)

The total soluble and insoluble-bound phenolics were determined in wood samples. The content of soluble phenolics in each tested wood variety decreased in the following order: Seedling date palm wood (SPW) > Old date palm wood (OPW) > Oak wood (OW) > Quibracho wood (QW) > Pinewood (PW) > Banana wood (BW). Extracts from BW had the lowest amount of soluble and bound phenolics, compared to other wood varieties (Table 4.1).

Phenolics appeared to be predominantly present in the soluble and insoluble-bound forms in the wood extracts. The soluble phenolics content of SPW, OPW, OW, QW, PW, and BW were 80.30, 74.65, 59.45, 43.34, 24.76, and 19.76 mg gallic acid equivalents (GAE)/ g dried wood, respectively as shown in Table 4.1. The bound phenolics were present in concentrations of 21.05, 19.89, 10.65, 12.41, 7.54 and 5.98 mg GAE/g dried wood, respectively. The results showed that SPW has the highest constituents of phenolic compounds, while BW has lowest

content. Dudonne *et al.* (2009) have studied 30 different wood plant species and measured their phenolic contents; they found that the *Myrocarpus fastigiatus* wood contained 119.14 ± 1.58 mg GAE/g of dried wood; *Quercus robur* wood also have 397.03 ± 0.05 mg GAE/g of dried wood, these results are similar to SPW in total soluble and insoluble-bound content. While the bark of *Pinus maritime* plant has 360.76 ± 0.04 mg GAE/g of dried wood, it should be mentioned that our results for PW in both soluble and insoluble-bound form were approximately 14.1 times lower than those reported by Dudonne *et al.* (2009). Some of the reported phenolics of *Acacia spp.* were gallic acid, caffeic acid, ferulic acid, and kaempferol (Singh *et al.*, 2008; Tung *et al.*, 2009).

Conde *et al.* (2014) measured the phenolic stilbenoids (pinosylvin and pinosylvin monomethyl ether) in the presence of both hexane and acetone-water extracts (in concentrations within the range of 0.17–0.62 and 0.38–3.31 g/100 g, respectively). The occurrence of pinosylvins in softwoods and/or their extraction has been reported in studies dealing with a number of spruce and pine wood samples (Willför *et al.*, 2003, 2007; Willför and Holmbom, 2004; Pietarinen *et al.*, 2006; Hovelstad *et al.*, 2006; Kokubo *et al.*, 1990; Geraldo de Carvalho *et al.*, 1996; Hillis and Inoue, 1968; Loman, 1970; Anderson, 1956; Simard *et al.*, 2008; Mahesh and Seshadri, 1954). In particular, the extraction of pinosylvins from *P. pinaster* wood by multistage operation (Alvarez-Novoa *et al.*, 1950; Hata, 1955) and by aqueous processing (Conde *et al.*, 2013) has been reported. The interest in pinosylvins is boosted by their biological properties, for example as anti-fungal, anti-bacterial, anti-listeria, anti-inflammatory or cytotoxic agents (Lindberg *et al.*, 2004; Lee *et al.*, 2005; Celimene *et al.*, 1999; Gref *et al.*, 2000; Bauerova *et al.*, 2011; Plumed-Ferrer *et al.*, 2013). The present investigation is the first study that evaluated the bound phenolics in wood. Furthermore, we were trying to gather details about the bound phenolics and their

antioxidant activities in by-products, to confirm their activities in the wood extracts that were selected for this study. Nicholson (1992), Waldron *et al.* (1996), Pang *et al.* (2018), Modafar *et al.* (2000), Balasundram *et al.* (2006), Nardini *et al.* (2002), Albishi *et al.* (2013a), Su *et al.* (2014), Dvořáková *et al.* (2008), Montilla *et al.* (2011), Ziegler *et al.* (2018), and Beatriz *et al.* (2014) have given a detailed account of the bound phenolics from different plants, food, and processing by-products.

4.2.2. Total flavonoid content (TFC)

Flavonoids have an important effect on the durability of wood (Chang *et al.*, 2001; Wang *et al.*, 2004). According to Schultz and Nicholas (2000), flavonoids protect heartwood against fungal colonization by a dual function: fungicidal activity and serving as excellent free radical scavengers (antioxidants). Flavonoids as natural antioxidants have received attention due to their role in the neutralization or scavenging of free radicals (Gupta and Prakash 2009). The hardwood of *Lonchocarpus castilloi* Standley (Leguminosae) showed high resistance to attack by termites. Other flavonoids isolated from the hardwood of (*Cryptotermes brevis*) showed feeding deterrent activity to *C. brevis* (Reves-Chilpa *et al.*, 1995).

In contrast, flavonoids were lower in their content in the wood extracts compared to non-flavonoid phenolics in this study; however, TFC was found to be highest in SPW than other woods. Sultana *et al.* (2007) and Feregrino-Pérez *et al.* (2011) registered the TFC as 2.14 ± 4.93 g catechin equivalents (CE) /100 g of dried wood. In this study, soluble SPW exhibited highest flavonoids content of 12.91 ± 0.10 mg CE/g of wood extract, followed by OPW > OW > QW > BW > PW, respectively (Table 4.1). The TFC in insoluble-bound phenolic extract of all wood extracts were very low compared to the soluble phenolics as shown in Table 4.1. The heartwood of *Acacia auriculiformis* (Leguminosae) contained a number of different flavonoids (Sarai *et al.*,

1980; Barry *et al*, 2005) that were highly resistant to attack by the dry wood termite *Cryptotermes brevis*. The heartwood of *Dalbergia congestiflora* Pittier (Leguminosae) tree showed natural resistance to fungal attack. The antifungal effect of various extracts from the *D. congestiflora* heartwood was evaluated against *Trametes versicolor* fungus (Martínez-Sotres *et al*, 2012).

Table 4.1. Total phenolic (TPC) and flavonoid (TFC) content of different woods¹

Wood sample	TPC (mg Gallic acid eq/g wood extract)		TFC (mg Catechin eq/g wood extract)	
	Soluble phenolics	Insoluble-Bound phenolics	Soluble flavonoids	Insoluble-Bound flavonoids
SPW	80.03 ± 4.47 ^a	21.05 ± 0.60 ^a	12.91 ± 0.10 ^a	1.75 ± 0.06 ^a
OPW	74.65 ± 2.62 ^b	19.67 ± 0.87 ^a	12.66 ± 0.39 ^a	1.68 ± 0.31 ^a
OW	59.45 ± 4.82 ^c	10.80 ± 0.10 ^b	9.32 ± 0.40 ^b	1.11 ± 0.06 ^b
QW	43.34 ± 0.72 ^c	12.41 ± 0.14 ^b	7.85 ± 0.03 ^b	0.99 ± 0.03 ^b
BW	19.76 ± 1.01 ^d	5.98 ± 0.01 ^c	3.98 ± 0.002 ^b	0.11 ± 0.00 ^c
PW	24.76 ± 0.26 ^d	7.54 ± 0.33 ^c	0.87 ± 0.08 ^c	0.25 ± 0.04 ^c

¹Data are expressed as means ± SD (n=3). Values with the same letter in each column are not significantly different (p > 0.05). SD: standard deviation, n is the number of observations in the sample.

4.2.3. Condensed and hydrolyzable tannin contents of wood extracts

Tannins constitute a distinctive and unique group of higher plant metabolites of relatively large molecular size (from 500 to >20,000 Da). They constitute one of the most important group of higher plant defensive secondary metabolites (Haslam, 1989). Condensed tannins are natural preservatives and antifungal agents, found in high concentrations in the bark and wood of some tree species (Zucker, 1983) while hydrolysable tannins are usually present in low amounts in plants (Nascimento *et al.*, 2013). The co-occurrence of both kinds of tannins in some plants or in some plant tissues is often observed (Hillis, 1987; Scalbert *et al.*, 1988). Furthermore, according to their structure, tannins offer different utilities (Roux *et al.*, 1980) and give to the plants their durability, among others (Hillis, 1987; Scalbert and Haslam, 1987). In addition, most plant-pathogenic fungi excrete extracellular enzymes such as cellulases and lignases, involved in the invasion and spread of the pathogen. Condensed tannins most likely act as inhibitors of these enzymes by complexion, thus blocking their action (Peter *et al.*, 2008). For this reason, extract from various woods and barks rich in tannin have been used as adhesives and wood preservatives for a long time (Brandt 1952; Plomely 1966; Mitchell and Sleeter 1980; Pizzi and Merlin 1981; Laks *et al.*, 1988; Lotz and Hollaway 1988; Toussaint 1997; Thevenon 1999). Cinnamon bark, which is frequently used as a spice, is an example of condensed tannin- rich plant. It is an ingredient in the traditional Kampo medicine prescription, which is also one of the official medicines in Japan.

In this study, two methods, known to afford the selective estimation of the two groups of tannins, are applied to wood extracts. The total of condensed tannins was evaluated using the HCl/n-butanol assay in both soluble and insoluble-bound forms. Meanwhile, the hydrolyzable tannins were measured by using the potassium iodate method as described in Chapter 3.

In this study, the soluble form of condensed tannins of SPW were the highest (103.19 ± 7.63 mg Cya eq /g of wood extract) compared to other wood extracts that were selected in this study (Table 4.2). In contrast, the insoluble-bound form was lower compared to the soluble content in all wood types; particularly, BW which showed the lowest content of condensed tannins in both soluble and insoluble-bound forms.

In contrast, the hydrolysable tannins were present in lower amounts in all wood extracts, compared to condensed tannins. Resistance to fungal invasion in wood is due to tannins, the ability of the hydrolysable tannins to remove metal cofactors through their strong affinity for metal ions (Mila *et al.*, 1996). Durable hardwoods such as oaks and chestnuts obtain much of their resistance through the deposition of ellagitannins, compounds in which gallic acid is attached via an ester bond to a D-glucose core and also biaryl-coupled to an adjacent galloyl group (Helm *et al.*, 1997). In this study, the SPW showed the highest content of soluble hydrolysable tannins (70.16 ± 5.16 mg of tannic acid/ g of wood extract) followed by OPW > OW > QW > PW > BW, respectively. In general, insoluble-bound form showed a lower content in all tested wood compared to soluble contents (Table 4.2).

So far, a relatively large number of compound-specific studies have investigated the hydrolyzable tannin composition of oak wood (Masson *et al.*, 1994; Viriot *et al.*, 1994; Conde *et al.*, 1998

Mosedale *et al.*, 1998; Fernandez de Simón *et al.*, 1999; Cadahia *et al.*, 2001). This interest has primarily been spurred by the needs of the wine industry, as most wine barrels are made of English oak (*Quercus robur*) or sessile oak (*Q. petraea*). Of the named hydrolyzable tannins, the heartwood of *Q. robur* has been found to contain castalagin, vescalagin, grandinin, and roburins (Hervé du Penhoat *et al.*, 1991; Masson *et al.*, 1994; Vivas *et al.*, 1995).

4.2.4. Lignin content of woods

Lignin, a cross-linked polymer with phenylpropane monomers, is the second most abundant biopolymer in nature (Mu *et al.*, 2018). It holds a great potential to be converted to high value-added phenolic platform chemical taking advantage of its abundant phenolic structure (Li *et al.*, 2015). Currently, 70 million tons of lignin are produced annually worldwide, yet only 5% is effectively converted to valuable chemicals, while the remaining 95% is primarily burnt to generate energy in the pulp and paper industry (Laurichesse and Averous, 2014; Mu *et al.*, 2015). Effective depolymerization of lignin is a great challenge that needs to be overcome so that lignin could be used as a value-added renewable resource available in massive quantities (Xu *et al.*, 2014). In addition, the structure and chemical composition of lignin vary in different plant species, or while using different extraction processes and subsequent treatments, thus increasing the complexity of lignin processing and decreasing its applicability in industrial processes (Ragauskas *et al.*, 2014; Constant *et al.*, 2016).

Table 4.3 presents the total amount of soluble and insoluble-bound lignin of each selected wood. The results showed that SPW has the highest amount of lignin in both forms, followed by QW> OPW> OW> PW> and BW, respectively. These results showed that the hardwood lignin is higher than softwood lignin in term of SPW, OPW, OW, and QW (hardwood), while PW and BW categorized as softwood. In agreement with our results, USDA (1971) indicated that the lignin composition of hardwood ranged from 65 to 85%, while softwood lignin ranged from 58 to 75%.

In disagreement with these results, Kebbi-Benkeder *et al.* (2014) found that knotwood species generally contained much higher contents of extractives in softwood than heartwood species. Other hardwoods have been investigated such as willow, acacia and poplar species (Pohjamo *et*

al. 2003; Pietarinen *et al.* 2005, 2006). Their knots were found to contain lower lignin contents than coniferous knots. However, hardwood knots contained higher quantities than the corresponding heartwood and flavonoids were detected as the main components. In our study, the acetyl bromide method presented higher recovery of lignin when compared with the other methods in all tissues evaluated. Besides, acetyl bromide method is simpler and faster than the other methods tested. Thus, it was suggested that the acetyl bromide method technique, among the techniques assessed in other studies is a preferable methodology to determine lignin content in wood (Moreira-Vilar *et al.*, 2014). Date palm wood is considered a renewable natural resource because it can be replaced in a relatively short period. It takes 4 to 8 years for date palms to bear fruit after planting, and 7 to 10 years to produce viable yields for commercial harvest. Usually date palm wastes are burned in farms or disposed in landfills, which cause environmental pollution in dates-producing nations. In countries like Iraq and Egypt, a small portion of palm biomass is used in making animal feed (Zafar, 2018). Our study concluded that SPW and OPW which are part of the family (*Phoenix dactylifera*) have high contents of lignin (70.16 ± 2.48 and 65.13 ± 5.22 mg lig/ g wood extract). They can therefore serve as a good renewable natural resource; particularly OPW, being an old tree can be used for this purpose instead of SPW, which will better serve to produce the date fruits. Thus, our studies on SPW and OPW to compare their composition of lignin have proven that we can use OPW instead of SPW due to their comparable contents of lignin.

Table 4.2. Condensed and hydrolyzable tannin contents of wood extracts¹

Wood Sample	Condensed Tannins (HCl/n-butanol) (mg Cya eq/g wood extract)		Hydrolysable Tannins (mg Tannic acid/g wood extract)	
	Soluble	Bound	Soluble	Bound
SPW	103.19 ± 7.63 ^a	44.06 ± 2.07 ^a	70.16 ± 5.16 ^a	46.39 ± 8.92 ^a
OPW	74.23 ± 2.65 ^b	21.45 ± 0.62 ^b	65.13 ± 4.71 ^b	27.88 ± 1.11 ^b
OW	50.82 ± 2.61 ^c	16.92 ± 7.23 ^c	0.03 ± 5.09 ^b	20.12 ± 1.29 ^b
QW	40.39 ± 2.09 ^c	9.98 ± 0.02 ^d	51.62 ± 2.03 ^c	10.95 ± 0.43 ^c
PW	31.01 ± 0.95 ^d	4.55 ± 1.11 ^d	40.45 ± 2.09 ^d	12.96 ± 1.33 ^c
BW	24.94 ± 1.190 ^d	1.99 ± 0.00 ^d	36.78 ± 0.95 ^d	7.89 ± 0.07 ^d

¹Data are expressed as means ± SD (n=3). Values with the same letter in each column are not significantly different (p > 0.05). SD: standard deviation, n is the number of observations in the sample.

Table 4.3. Lignin contents of wood extracts¹

Wood sample	Lignin content (mg Lig eq/g wood extract)	
	Soluble	Insoluble-bound
SPW	70.16 ± 2.48 ^a	28.39 ± 4.75 ^a
OPW	65.13 ± 5.22 ^b	27.88 ± 1.08 ^a
OW	60.03 ± 2.60 ^b	20.12 ± 0.71 ^b
QW	66.45 ± 3.95 ^b	20.56 ± 0.73 ^b
PW	41.62 ± 5.29 ^b	13.25 ± 2.97 ^c
BW	36.78 ± 3.41 ^c	2.96 ± 2.91 ^d

¹Data are expressed as means ± SD (n=3). Values with the same letter in each column are not significantly different (p > 0.05). SD: standard deviation, n is the number of observations in the sample.

4.3. Determination of the effect the antioxidant activities of SP and ISP extracted from wood varieties

Many analytical methods are used to measure the antioxidant activity of substances, yet little is known about the comparability of the tested results among different methods. Previously reported research has shown that one single method can hardly reflect comprehensive antioxidant capacity generated by a series of complex compounds in the plant, because different antioxidant compounds may act through distinct mechanisms against oxidizing agents (Marazza *et al.*, 2012; Xiao *et al.*, 2014). For this reason, different antioxidant assays were employed to detect the antioxidant capacities of SPW, OPW, OW, QW, PW and BW. Among these methods, DPPH scavenging activity, trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and iron chelating assays are all chemical methods, while DNA, LDL, and α -glucosidase inhibitory activity are the biological antioxidant assays.

4.3.1. Measurement of total antioxidant capacity by trolox equivalent antioxidant capacity (TEAC) assay

Trolox equivalent antioxidant capacity (TEAC) assay was first developed as a simple and convenient method for total antioxidant capacity (TAC) determination (Miller *et al.*, 1993). The assay measures the ability of antioxidants to scavenge the stable radical cation ABTS $^{\bullet+}$ (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), a blue-green chromophore with maximum absorption at 734 nm that decreases in its intensity in the presence of antioxidants. Antioxidants can neutralize the ABTS radical cation (ABTS $^{\bullet+}$) generated from ABTS, by either direct reduction via electron donation or by radical quenching via hydrogen atom donation, and the balance of these two mechanisms is generally determined by antioxidant structure and pH of the medium (Prior *et al.*, 2005). TEAC assay has been used to measure the total antioxidant activity

of pure substances, body fluids, and plant materials. TEAC assay, similar to other radical scavenging methods, can be automated and adapted to microplates and flow injection techniques (Milardovic *et al.*, 2007). In this study, scavenging assays with respect to antioxidant activity showed a decrease in activity in the order of SPW > OW > OPW > QW > BW > PW. The antioxidant activity of SPW (15.37 ± 0.24 mmoles trolox eq/g of wood extract) was 2.09 times higher than those of other selected woods (Table 4.4). The phenolic compounds present in SPW and other wood extracts may act by quenching of free radicals.

4.3.2. DPPH radical scavenging capacity (DRSC) using electron paramagnetic resonance (EPR)

Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals is among the most frequently used assays that offers the first approach for evaluating antioxidant activity. DPPH is a stable chromogen radical with a deep purple colour. It is commercially available and does not need to be generated prior to the assay. Phenolic antioxidants neutralize the DPPH radical which is accompanied by a colour change that is measured at 517 nm. The DPPH assay is a simple technique and requires only a UV spectrophotometer or an EPR spectrometer.

As it can be seen from the Table 4.5, both the soluble and insoluble-bound extracts of SPW, OPW, OW, QW, PW, and BW were capable of scavenging DPPH radicals. There was no statistically significant ($P < 0.05$) difference between SPW extract and OPW. PW and BW showed lower activity than others, and hence a significant difference was noted. The soluble phenolics of SPW (15.65 ± 0.51 mmoles trolox eq/g wood extracts) showed the highest activity compared to other selected woods (Table 4.5).

The DPPH radical scavenging capacity of bound phenolics of wood extracts ranged from 8.53 ± 1.02 to 1.88 ± 0.03 (mmoles trolox eq/g wood extract) for DPPH. Among SPW, OPW, OW, QW, PW, and BW, the antioxidant capacity of the insoluble-bound phenolic extract of OPW was higher than the other selected woods (Table 4.5). Liyana-Pathirana and Shahidi (2006) reported that the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free phenolics in hard and soft whole wheats. Adom and Liu (2002) reported that the bound phenolics were the major contributors to the total antioxidant activity in cereals. Taken together, our study supported the conclusion of Liyana-Pathirana and Shahidi (2006) who suggested that it is essential to include the bound phenolics in studies related to quantification and antioxidant activity evaluation of grains and cereals.

Table 4.4. Antioxidant capacity (TEAC) of extracts from dried frozen wood¹

Wood sample	TEAC (mmoles trolox eq/g wood extract)	
	soluble phenolics	bound phenolics
OPW	12.42 ± 0.82 ^a	3.34 ± 0.05 ^a
SPW	15.37 ± 0.24 ^a	3.40 ± 0.76 ^a
OW	14.23 ± 0.24 ^a	1.82 ± 0.12 ^b
PW	2.94 ± 0.005 ^b	0.12 ± 0.008 ^c
QW	12.33 ± 3.23 ^a	1.06 ± 0.009 ^b
BW	7.73 ± 1.77 ^b	0.19 ± 0.003 ^c

¹Data are expressed as means ± SD (n=3). Values with the same letter in each column are not significantly different (p > 0.05). SD: standard deviation, n is the number of observations in the sample.

Table 4.5. DPPH radical scavenging capacity (DRSC) using (EPR)¹

Wood sample	DPPH (mmoles trolox eq/g wood extract)	
	Soluble phenolics	Bound phenolics
SPW	15.65 ± 0.51 ^a	6.43 ± 1.19 ^a
OPW	14.18 ± 0.69 ^a	8.53 ± 1.02 ^a
OW	12.45 ± 0.46 ^a	7.07 ± 0.16 ^a
PW	10.50 ± 0.06 ^b	5.85 ± 0.13 ^a
QW	13.58 ± 0.64 ^a	2.30 ± 0.06 ^b
BW	8.44 ± 0.27 ^c	1.88 ± 0.03 ^b

¹Data are expressed as means ± SD (n=3). Values with the same letter in each column are not significantly different (p > 0.05). SD: standard deviation, trolox eq (trolox equivalents); n is the number of observed samples.

4.3.3. Ferric reducing antioxidant power (FRAP) assay

The total antioxidant activity can be measured by the ferric reducing antioxidant power assay (FRAP). The flavonoids and phenolic acids are present in the plants exhibit strong antioxidant activity, which is dependent on their potential to form the complex with metals such as iron and copper. This method is based on the principle of increase in the absorbance of the reaction mixtures with antioxidant activity (Vijayalakshmi and Ruckmani, 2016). The antioxidant compound present in the samples forms a coloured complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700 nm by UV-spectrophotometer. In this study, the effects of SPW, OPW, OW, QW, PW, and BW wood extracts on antioxidant activity of samples were evaluated using the FRAP method. The reductive capabilities were found to increase with increasing concentration of each sample (mmoles trolox eq/g wood extract), and the results are given in Table 4.6. The ferric reducing assay of the soluble phenolics of SPW suggest that it has the potential to reduce the ferric form. The absorbance values were increased with increased concentration of the extract. From the study, it can be concluded that the soluble extract of selected woods possesses antioxidant activity. According to the single-electron transfer-based method FRAP assays, the values for the soluble SPW, OPW, QW, PW, OW, and BW extracts were 1.37 ± 0.02 , 1.18 ± 0.32 , 1.15 ± 0.27 , 0.59 ± 0.14 , 0.55 ± 0.04 , 0.35 ± 0.03 , respectively.

In this assay the yellow colour of the test solution changes to various shades of green and blue, which in turn depends upon the reducing power of each compound? The presence of radicals (ie antioxidants) causes the conversion of the Fe^{3+} / ferricyanide complex used in this method to the ferrous form. A higher absorbance at 700nm indicates a higher reducing power. The results of the reducing power assay are given in Table 4.6. The higher absorbance of soluble extracts may

be due to its strong reducing power potential compared to insoluble-bound extracts which showed lower reducing power activity. Comparing SPW and OPW extracts, SPW extracts showed better reducing power than OPW extracts. The reducing power of the extracts may be due to the biologically active compounds in the extract which possess potent electron donating abilities. This assay further confirmed the antioxidant properties of the extracts.

4.3.4. Measurement of iron (II) chelation capacity

Transition metal ions are known to stimulate lipid oxidation by the Fenton reaction and by decomposing lipid hydroperoxides into more reactive peroxy and alkoxy radicals. Further, some phenolics and flavonoids, known as antioxidants, are powerful metal chelators. The antioxidants can easily deactivate prooxidant metal ions and, thus, prevent or retard metal ion-induced lipid oxidation. The antioxidant property of metal chelators is assessed when a complex is formed between the antioxidant and the metal, in such a way that metal ions can no longer act as an initiator of lipid oxidation. Therefore, metal chelation capacity is also used as indicator of antioxidant activity, usually in combination with other antioxidant assays. Metal chelation capacity has been investigated for a number of antioxidants and extracts (Chandrasekara and Shahidi, 2010; Karawita *et al.*, 2005; Wettasinghe and Shahidi, 2002; Wijeratne *et al.*, 2006; Zhong *et al.*, 2012).

Adjacent phenolic groups or special arrangement of carbonyl and hydroxyl groups in a molecule may act as chelator or metal binder. Thus a deprotonated hydroxyl group that carries a high charge density oxygen centre and located next to another oxygen center such as hydroxyl group in the *ortho* position act as a good chelator (Hider *et al.*, 2005). In this assay, a decrease in the intensity of ferrozine–ferrous colour complex (pink colour) by wood phenolic extracts was measured at 562 nm using a spectrophotometer and reported as EDTA equivalents (Table 4.6).

Metal chelation values of soluble and insoluble-bound of SPW, OPW, OW, QW, PW, and, BW varied between 554.34 and 2117.52 $\mu\text{mol EDTA equiv}/100\text{g}$ sample. SPW (2117.52 ± 144.79 $\mu\text{mol EDTA equiv}/100\text{g}$) had the highest ($p < 0.05$) metal chelating ability followed by OPW (1082.05 ± 160.26 $\mu\text{mol EDTA equiv}/100\text{g}$), OW (1068.38 ± 133.60 $\mu\text{mol EDTA equiv}/100\text{g}$), QW (986.88 $\mu\text{mol EDTA equiv}/100\text{g}$), PW (986.88 ± 155.40 $\mu\text{mol EDTA equiv}/100\text{g}$) SPW and OPW also had the highest total phenolic content among others. However, insoluble-bound extracts show lower chelating ability. This could be due to the amount and type of phenolic compounds present in the insoluble-bound fraction. All other wood extract fractions showed metal chelating ability, except BW, which showed the lowest ability. Mention should be made that metal chelation of insoluble-bound phenolics of wood extracts is being reported here for the first time.

Table 4.6. Antioxidant capacity (reducing power and iron chelating) of wood extracts¹

Wood Sample	Reducing Power (mmoles trolox eq/g wood extract)		Iron chelating activity (μmoles EDTA eq/100 g wood extract)	
	Soluble phenolics	Insoluble-Bound phenolics	Soluble phenolics	Insoluble-Bound phenolics
SPW	1.37 ± 0.02 ^a	0.81 ± 0.03 ^a	2117.52 ± 144.79 ^a	1295.73 ± 185.05 ^a
OPW	1.18 ± 0.32 ^a	0.13 ± 0.004 ^d	1082.05 ± 160.26 ^b	1142.31 ± 167.57 ^b
OW	0.55 ± 0.04 ^b	0.30 ± 0.008 ^c	1068.38 ± 133.60 ^b	1028.63 ± 244.80 ^c
PW	0.59 ± 0.14 ^b	0.70 ± 0.05 ^b	694.44 ± 165.05 ^c	308.12 ± 44.60 ^d
QW	1.15 ± 0.27 ^a	0.68 ± 0.007 ^b	986.88 ± 155.40 ^b	859.43 ± 74.80 ^c
BW	0.35 ± 0.03 ^c	0.10 ± 0.004 ^d	554.34 ± 125.08 ^c	228.16 ± 24.86 ^d

¹Data represent mean values ± standard deviation (n = 3). Values followed by the same letters within a column are not significantly different (p > 0.05). Standard deviation, EDTA eq (Ethylenediaminetetraacetic acid equivalents); trolox eq (Trolox equivalents), n is the number of observed samples.

4.4. Evaluation of antioxidant activity of wood extracts in biological model systems

Phenolic compounds are widely distributed in plants and their antioxidant activity and free radical-scavenging ability as well as their potential beneficial health effects have been of much research interest (Ross and Kasum, 2002). When added to foods, antioxidants minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality, and increase shelf life of products (Jadhav *et al.*, 1996). These antioxidants may help to relieve oxidative stress and preventing free radicals from damaging biomolecules such as proteins, DNA, and lipids (Shahidi and Naczki, 2004b). The antioxidant activity of extracts of several plants, including their leaves, bark, wood, roots, fruits, and seeds has been extensively studied (Mariod *et al.*, 2008). However, many researchers have also reported the adverse effects of

synthetic antioxidants such as toxicity and carcinogenicity. Natural antioxidants are in high demand for application as nutraceuticals, bio-pharmaceuticals, as well as food additive because of consumer preference.

Phenolic compounds provide essential functions in the reproduction and growth of plants, act as defense mechanisms against pathogens, parasites, and predators, as well as contributing to the colour of plants (Wink, 2003). In addition, phenolics abundant in plants are reported to play an important role as chemopreventive agents; for example, the phenolic components of onion and potato by-products have been linked with anticarcinogenic/antimutagenic in *in-vitro* (Albishi *et al.*, 2013b). Many phenolic compounds have been reported to possess potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities to varying extent. Recent studies have characterized a large number of natural phenolic compounds from food processing by-products (Ambigaipalan *et al.*, 2016).

4.4.1. Inhibition of cupric ion-induced human low-density lipoprotein (LDL) peroxidation

Oxidative modification of LDL-cholesterol plays a key role in the pathogenesis of atherosclerosis and heart disease (Sajilta *et al.*, 2008). Oxidative LDL containing both lipid oxidation products and oxidized apoprotein particles contribute to all stages of the atherosclerosis process, thus promoting the atherogenic effects (Shahidi, 2015; Shahidi and Zong, 2010, 2015). Antioxidants such as many polyphenols can inhibit LDL oxidation by scavenging free radicals, chelating prooxidant metals, and binding with lipoprotein B, which promotes the access of antioxidant to the lipids and prevents the approach of oxidation catalysts (Satue-Gracia *et al.*, 1997); thus, inhibiting LDL oxidation may reduce these risks (Schnitzer *et al.* 1997; Jeong *et al.* 2004). Antioxidants are potential anti-atherogenic agents in cardiovascular

disease risk reduction, and their potencies can be assessed by LDL-cholesterol oxidation inhibition assay. Polyphenols, including procyanidins, have been reported to have antioxidant activity (Koga *et al.* 1999), antiulcer activity (Saito *et al.* 1998), inhibition activity on cariogenic factors (Yanagida *et al.* 2000), and are also known to promote hair epithelial cell growth (Takahashi *et al.* 1999). Since they are reported to have antioxidant activity, the phenolic extracts from SPW, OPW, OW, QW, PW, and BW were evaluated for their inhibition activity on low-density lipoprotein (LDL) oxidation.

The LDL-cholesterol oxidation inhibition assay has been used by many researchers for assessing antioxidant activity and potential anti-atherogenic effect of various antioxidant compounds and extracts (Chandrasekara and Shahidi, 2011; Madhujith and Shahidi, 2007; Zhong and Shahidi, 2012).

In this study, the oxidation of LDL-cholesterol was induced by cupric ion (Cu^{2+}). The assay of oxidation for LDL was measured by the formation of conjugated dienes, which is measured spectrophotometrically at 234 nm. Absorbance values for the formation of conjugated dienes measured up to 25 h for SPW, OPW, OW, QW, PW, and BW (soluble and bound) during cupric ion-induced human LDL oxidation is shown in Figure 4.1. Among all of the tested samples, BW extracts showed the lowest inhibitory effect against LDL oxidation. This could be due to the poor chelating ability of cupric ions, hence promoting oxidation (Table 4.6) . The LDL oxidation inhibitory activity of soluble wood extracts followed the order $\text{SPW} > \text{OPW} \sim \text{OW} > \text{QW} > \text{PW}$, promising inhibitory activity against cupric ion-induced LDL oxidation. This implies the importance of including the insoluble-bound fraction from wood components in the analysis. In addition, it is evident (Figure 4.2) that the formation of conjugated dienes was stabilized after 12 h of incubation during 25 h of assay period. Vuorela *et al.* (2005) reported that the higher amount

of phenolics in pine bark extract facilitates higher LDL oxidation inhibition 86% compared to the raspberry extract. In addition, Hu and Kitts (2007) suggested that the greater the lipophilic property of the antioxidant, the more effective it will be in extending the LDL oxidation lag phase once induced by Cu^{2+} . The LDL oxidation inhibition effect of wood extracts could be attributed to their tannin content. Similarly, Park *et al.* (2014) showed that the procyanidins from the stem wood of *Machilus japonica*, extracted with 80 % aqueous MeOH, has very high inhibition effect against LDL oxidation, between 7.6 - 97.6%. Huh *et al.* (2011 and 2012) reported that procyanidin from hardwood of *Lindera umbellata* and *Lindera fruticosa*, is an inhibitor of LDL-oxidation. In addition, the antioxidant activities of the *L. glauca* hardwood were evaluated against LDL-oxidation, and results confirmed that it efficiently counteracts LDL-oxidation *in vitro* (Huh *et al.*, 2014). Phenolic acids as well as the lignans pinoresinol and matairesinol extracted from pine bark may be consider as being safe for possible food applications, including functional foods intended for health benefit. Because the mechanism of LDL oxidation inhibition by phenolic compounds is still unclear, it is difficult to ascertain a confirmed reason for the inhibition mechanism involved. This study also revealed that not all extracts from wood could exert inhibitory action against LDL oxidation, such as BW. In general, all soluble phenolic extracts tested here showed high inhibition activity against LDL oxidation compared to the insoluble-bound form, which has been related to the risk of coronary heart disease. Therefore, these results provide useful information for the future biomedical use of these phenolics as pharmaceutical agents.

In this study, SPW and OPW contained high amounts of polyphenols including flavonoids, which constitute the largest and most studied group of plant phenolics. Flavonoids are powerful antioxidants and their activity is related to chemical structures (Rice-Evan *et al.*, 1995; Rice-

Evan *et al.*, 1996). Plant phenolics and flavonoids are multifunctional and can act as reducing agents, as hydrogen atom-donating antioxidants, and as singlet oxygen quenchers. Certain flavonoids also act as antioxidants via their metal ion chelation properties (Brown *et al.*, 1998), thereby reducing the metal's capacity to generate free radicals. Phenolic acids and flavonoids can act as potent inhibitors of LDL oxidation via several mechanisms including protection of the LDL-associated antioxidant α -tocopherol (vitamin E). The protection of LDL against copper ion or free radical-induced oxidation by phenolics depends on their response to copper ion, their partitioning between the aqueous and the lipophilic compartments within the LDL particle, and their hydrogen donating antioxidant properties (Brown *et al.*, 1998).

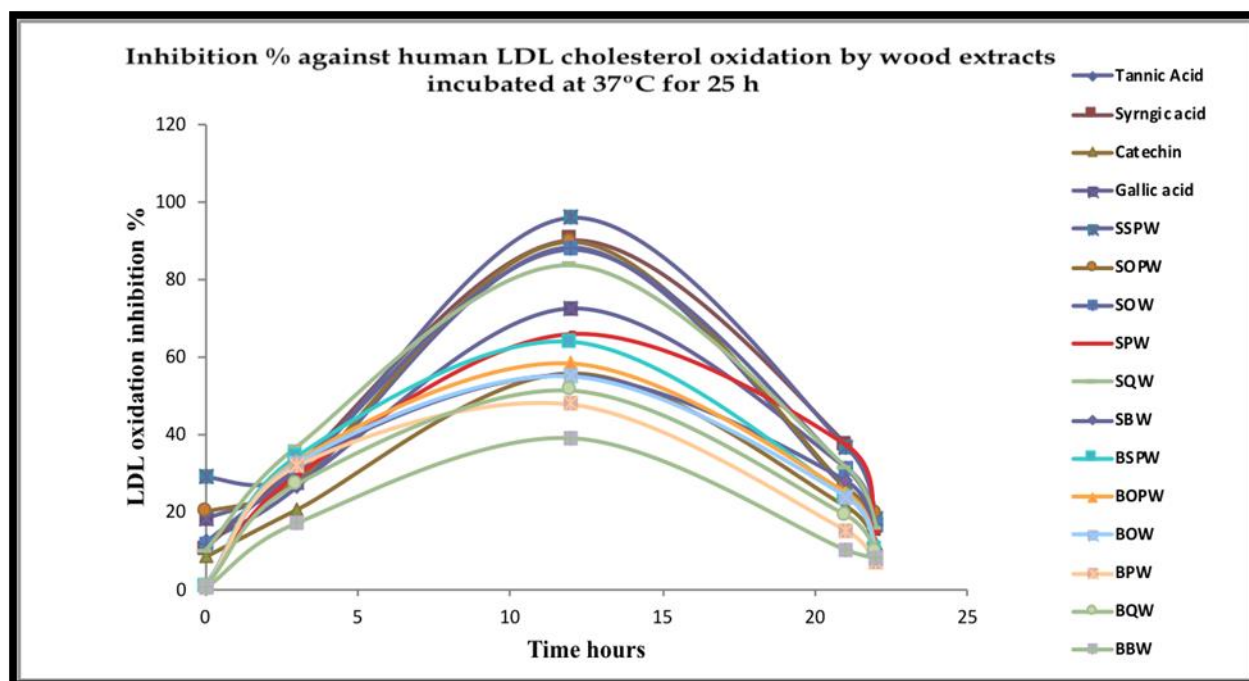


Figure 4.1. Inhibition of cupric ion-induced human low density lipoprotein (LDL) cholesterol oxidation by Soluble and insoluble-bound wood extract. LDL (0.2 mg of 0.1 mg protein/mL) was oxidized in PBS (pH 7.4) at 37°C with 4 μ M CuSO₄, and absorbance was continuously monitored at 232 nm. Each line shows the individual % of different soluble and insoluble-bound samples.

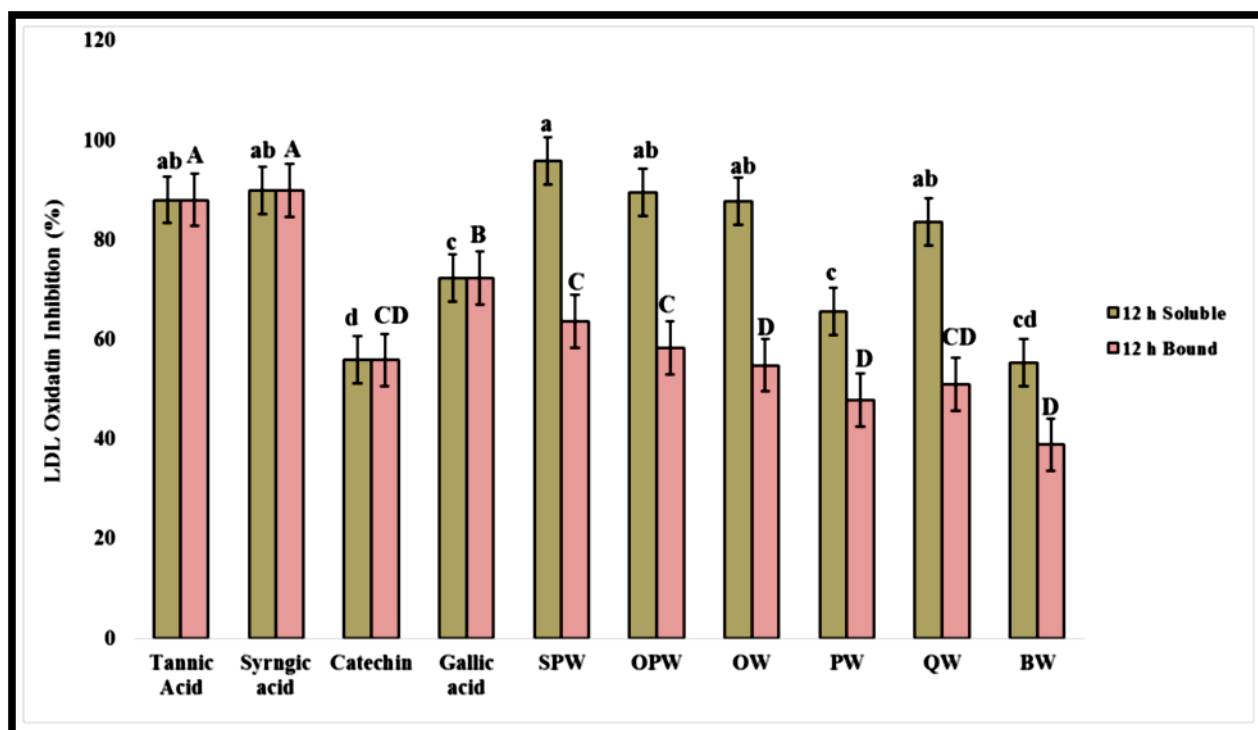


Figure 4.2. Inhibition % against human LDL cholesterol oxidation by wood extracts incubated at 37 °C for 12 hours

4.4.2. Supercoiled strand DNA scission by peroxy and hydroxyl radicals

Cancer is a growing health problem around the world and is the second leading cause of death after heart disease (Reddy *et al.*, 2003). There are now more cases of cancer per year worldwide, including a group of more than 100 diseases such as cancer of the liver, lung, stomach, colon, breast, and so forth (Surh, 2003; Luk *et al.*, 2007). There is no effective drug to treat most cancers. There is a general call for new drugs that have a minor environmental impact, and are highly effective, which could prevent approximately 30-50% of cancers. Novel natural products offer opportunities for innovation in drug discovery (Cai *et al.*, 2012). Many clinical trials on the use of nutritional supplements and modified diets to prevent cancer are ongoing (Greenwald, 1996).

Oxidative stress in cells also leads to DNA damage implicated in mutagenesis and carcinogenesis, and other pathological processes as well as the aging process. Oxidative damage to the DNA may occur at both the phosphate backbone and the nucleotide bases, resulting in a wide variety of modifications, including strand scission, sister chromatid exchange, DNA-DNA and DNA-protein cross-links as well as base modification (Davies, 1995). Many polyphenols and extracts have been evaluated in DNA model systems for their potential as antioxidants and antimutagenic agents in cancer prevention and/or treatment (Chandrasekara and Shahidi, 2011; Zhong and Shahidi, 2012).

The effect of antioxidants on free radical-induced DNA strand scission has been determined in a supercoiled plasmid pBR322 DNA model system, where DNA strand breaking is induced by hydroxyl or peroxy radicals (Chandrasekara and Shahidi, 2011; Zhong and Shahidi, 2012). These biologically relevant reactive oxygen species (ROS) are responsible for oxidative damage to DNA, especially the mitochondrial DNA (Perron *et al.*, 2008). The hydroxyl radical can be

generated by the Fenton reaction between ferrous ion and hydrogen peroxide, while the peroxy radical is generated by AAPH. After incubation with radicals and antioxidants at 37°C, the DNA fractions are separated by gel electrophoresis and bands identified after visualization. Both the open circular form of the DNA from single strand cleavage and linear form from double strand cleavage may be observed as a result of DNA oxidation, with the open circular form being dominant. The concentration of the native (supercoiled) and nicked DNA fractions is obtained from densitometer as indicated by the intensity or density of the corresponding bands. Antioxidants inhibit DNA scission possibly through a combination of radical scavenging and ferrous ion chelation mechanisms, and their inhibition efficiency can be calculated as DNA retention (percent of DNA retained unoxidized and supercoiled).

The DNA oxidation inhibition assay provides valuable information on the efficacy of antioxidants in protecting DNA from oxidative stress, which may suggest their antimutagenic and anticarcinogenic potentials.

The hydroxyl radical is highly reactive and could easily react with biomolecules (amino acids, proteins, enzymes, RNA, and DNA), hence leading to cell or tissue injury associated with degenerative diseases (Shahidi and Ambigaipalan, 2015; Ambigaipalan *et al.*, 2015; Amarowicz *et al.*, 2004). Hydroxyl radicals generated in an $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ system by Fenton reaction will form a spin adduct of 5,5-dimethyl-1-pyrroline N-oxide (DMPO), which could be reflected in a typical EPR signal (1:2:2:1). The height of the third peak of the EPR spectrum represents the relative amount of DMPO–OH adducts that could be decreased in the presence of antioxidants (Je *et al.*, 2005). Hydroxyl radical scavenging activity of wood extract components was expressed as $\mu\text{mol/g}$ tannic acid equivalents (TA), and the value ranged between 9.04 ± 0.63 and 38.64 ± 0.74 $\mu\text{mol TA/g}$ sample (Table 4.7). OPW exhibited the highest hydroxyl radical scavenging activity,

whereas PW showed the lowest activity. This assay also showed a similar trend as other antioxidant assays explained above. A few studies have shown hydroxyl radical scavenging activity of wood. Sathya and Siddhuraju (2012) showed that the phenolics extracted from bark of empty pod have registered the protective effect (87.60 ± 6.84 %) against hemolysis of human RBC's induced by AAPH. It is comparable with the standards BHA (91.06 ± 2.41 %) and tannic acid (89.91 ± 0.87 %).

In the present study, soluble and insoluble-bound phenolics of wood extracts were evaluated for their capacity in inhibiting peroxy and hydroxyl radical-induced DNA supercoiled (form I) strand scission. Figures 4.3-A and 4.4-B show the activity of soluble wood extracts for inhibiting peroxy and hydroxyl radical-induced DNA supercoiled (form I) strand scission.

The DNA molecule can easily be attacked by free radicals that induce base modification and strand scission; which would lead to mutagenesis and possibly cancer. Thus, the effectiveness of the extracts to prevent the scission of the DNA strands is a reflection of their positive effects against many diseases in the biological systems. Peroxy radical, which has been used in the present study, is known to exert oxidative damage in biological systems due to its comparatively long half-life and thus greater affinity to diffuse into biological fluids in cells (Hu and Kitts, 2001). Soluble extracts from wood varieties were dissolved in 10 mM PBS before mixing them with the DNA. Figure 4.3-A shows the percentage of supercoiled DNA strands retained after incubation with peroxy radicals generated by AAPH. Soluble phenolic extracts from SPW were most effective showing a DNA strand scission inhibition of 98.42% followed by OPW (86.39%), OW (69.41%), QW (89.06%), BW (77.89%), while extracts from PW showed a low activity of approximately 51.85% (Figure 4.3-A). Tannic acid was used as a positive standard and showed 87.93% inhibition of DNA. The tannic acid activity against peroxy radical was lower than that

of SPW which showed the strongest inhibition compared to tannic acid and other samples tested in this study.

Radicals cleave supercoiled pBR 322 plasmid DNA (form I) to nicked circular DNA (form II) as shown in Figure 4.3-A. Lane 1 represents the native DNA without AAPH and antioxidant additives and lane 2 represents the blank, where the reaction mixture does not contain any antioxidant. The presence of a high intensity form II (nicked) band and the disappearance of form I (supercoiled) band in lane 2 indicate that the DNA was completely nicked. The wood extracts which were added in the remaining wells showed good strand scission inhibiting activity as already described. In the absence of any antioxidant, it may be expected that the peroxy radical abstracts a hydrogen atom from the nearby DNA to generate a new DNA radical, which in turn evokes a free radical chain reaction resulting in the cleavage of the DNA molecule. However, in the presence of antioxidants, this chain reaction is terminated by abstracting a hydrogen atom from the antioxidant molecule (Hu and Kitts, 2000).

In the present study, soluble wood extracts exhibited inhibition of hydroxyl radical- induced DNA nicking in both site-specific and non-site-specific models. The concept of site-specific effect of hydroxyl radical was described by Gutteridge (1984). In the absence of EDTA, iron ions bind to deoxyribose molecules and bring about a site-specific reaction in the molecule. However, in the presence of EDTA, iron ion is removed from binding site to form EDTA metal complex and produce hydroxyl radical that can be removed by hydroxyl radical scavenging.

Wood extracts showed radical scavenging and antioxidant activities. The wood extracts have phenolic hydroxyl groups in their structures and these have been recognized to function as electron or hydrogen donors (Shahidi and Wanasundara, 1992). The antioxidants have attracted much interest with respect to their protective effect against free radical damage that may be the

cause of many diseases, including cancer (Nakama *et al.*, 1993). The antioxidative effect of wood extract is mainly due to its phenolic components, such as flavonoids (Pietta *et al.*, 1998). Some flavonoid and non-flavonoid compounds have been reported to show alkyl and peroxy radical scavenging activity, thus reducing radical-mediated pathogenesis, e.g. carcinogenesis (Sawa *et al.*, 1999).

When peroxy or hydroxyl radical oxidizes DNA, the supercoiled form may be converted to a nicked open circular form followed by a linear form. Supercoiled DNA moves more rapidly through an agarose gel network in comparison with the linear form of DNA. Thus, we could observe two rows of DNA, namely nicked (N) and supercoiled (S) (Figure 4.3-A, B). Areas of these bands were used to calculate the inhibition percentage by wood extracts. The inhibitory activity of peroxy- and hydroxyl-induced DNA strand scission, of the selected wood (soluble form), ranged from 51.85 to 98.42% and from 19 to 38 %, respectively.

Hydroxyl radical generated by Fenton reaction is well known to cause oxidative induced breaks in DNA strands to yield its open circular or relaxed forms. At a concentration of 20µg/mL, free radical scavenging effect of 70% methanol-acetone-water solvents of soluble phenolic extracts of different woods were studied (Figure 4.3-B) on plasmid DNA damage. The extracts of soluble SPW and OPW (lanes 3 and 4) showed a significant reduction in the formation of nicked DNA and increased native form of DNA. The protection offered by QW (lane 7) extract was close to that of OPW extract (lane 4). The OW (lane 5) and PW (lane 9) showed moderate, while PW (lane 6) showed comparatively low protection. The OPW and SPW extracts with high phenolic content showed better protection (%) in term of both hydroxyl and peroxy radicals compared to the other selected wood (Table 4.7), indicating that protection was directly proportional to the concentration of total phenolics present. Polyphenols are potential protecting agents against the

lethal effects of oxidative stress and offer protection to DNA by chelating redox-active transition metal ions.

Wood is widely used all over the world however, 40% amount of OPW considered as waste after producing the dates. In the present study, it was found that SPW and OPW were rich sources of phenols with promising antioxidant and free radical scavenging activities and ability to provide protection against DNA damage caused by reactive oxygen species (ROS). Thus, they may be used in order to protect them from oxidized damage and possibly in supplements to render health benefits in term of antimutagenic agents in cancer prevention and/or treatment.

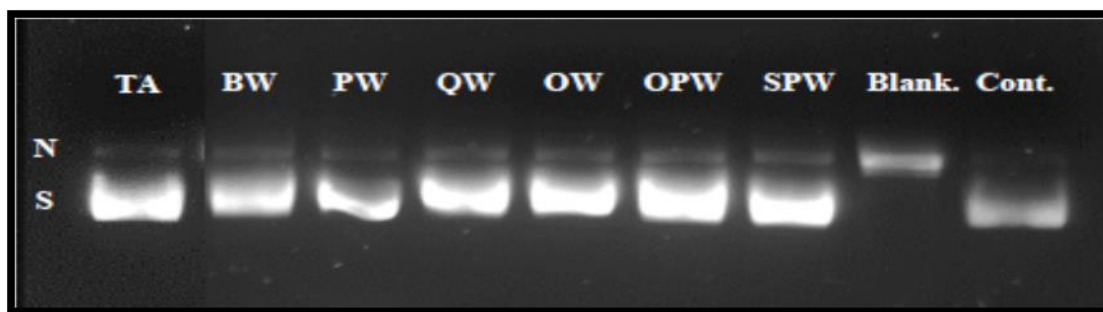


Figure 4.3-A. Agarose gel electrophoresis of inhibition of peroxyl radical-induced DNA scission by soluble wood extracts and tannic acid; peroxyl radical generated by induced AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride).

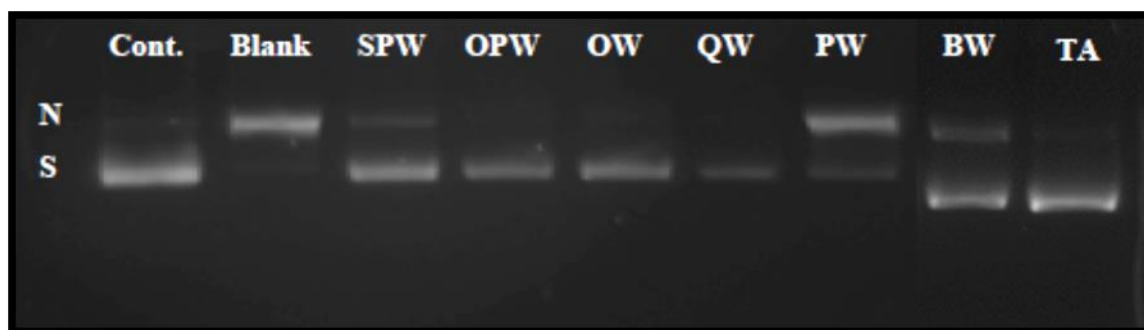


Figure 4.3-B. Agarose gel electrophoresis of inhibition of hydroxyl radical-induced DNA scission by soluble wood extracts and tannic acid; hydroxyl radical generated between H_2O_2 and ferrous ion. N (nicked), S (supercoiled) DNA.

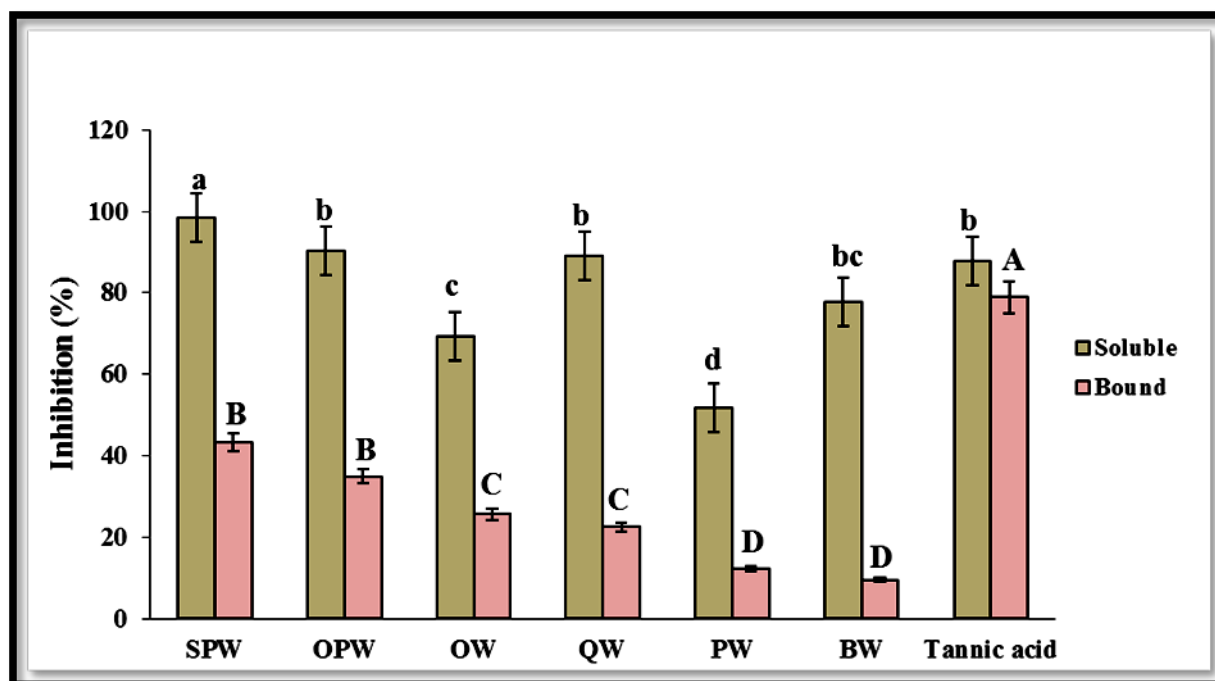


Figure 4.4. Inhibition (%) of effect the addition of soluble and insoluble-bound wood phenolic extracts in peroxy radical treated DNA system. *All data represent the mean of triplicates, values followed by the same symbols are not significantly different ($p>0.05$) by Tukey's HSD test

Table 4.7. Inhibition (%) of DNA strand section of extracted wood phenolics by hydroxyl and peroxy radicals¹

Wood sample	DNA scission inhibition (%)	
	Hydroxyl radical	Peroxy radical
SPW	36.54±2.39 ^a	98.42±0.91 ^a
OPW	38.64 ±0.74 ^a	86.39±1.94 ^b
OW	29.85±1.93 ^b	69.41±1.97 ^c
PW	19.05±0.63 ^c	51.85±2.66 ^c
QW	27.07±3.69 ^b	89.06±0.89 ^b
BW	24.88±4.66 ^b	77.89±2.13 ^c

¹All data represent the mean of triplicates, values followed by the same symbols are not significantly different ($p>0.05$) by Tukey's HSD test

4.4.3. α -Glucosidase inhibitory activity

High blood sugar level is one cause of diabetes due to the disruption of a metabolic system of carbohydrates, proteins and fats resulting in complications of the kidneys, eyes and cardiovascular system (Oyedemi *et al.*, 2011, Patel *et al.*, 2012, Zhang and Li, 2014). The effect of these complications can lead to the death of diabetic patients. Diabetes is the third cause of the death after cancer, cardiovascular and cerebrovascular diseases (Patel *et al.*, 2012). The number of diabetic patients in the world in 2014 was 386.7 million people with the prevalence level 8.3%, and this will increase up to 591.9 million people in 2030 (International Diabetes Federation, 2014). According to International Diabetes Federation (2014), the prevalence of diabetic patient above 15 years old in 2013, was 2.1%, increasing two-fold compared with that in 2007; diabetes is the sixth cause of death. Diabetes type 2 (non-insulin-dependent diabetes mellitus) is most dominant; almost 90% of all diabetic cases belong to this type in the world (Hasan *et al.*, 2014). This condition is grave as the pancreas needs serious treatment to solve this problem. One of the ways that can be used as a therapeutic approach in the treatment of diabetes is postprandial control of blood sugar levels by delaying of the glucose absorption. α -Glucosidase enzymes in the epithelial mucosa of small intestine have a function to break up the complex carbohydrates in the glycoside bonds to make mono- and disaccharides such as maltase maltose and sucrose that can be absorbed by intestinal epithelial cells. Inhibition of α - glucosidase in the digestive system will delay the digestion of carbohydrates and extends the digestion time which decreases the rate of glucose absorption resulting in the reduction of blood glucose level. The inhibition of α -glucosidase is therapeutic for patients with type 2 diabetes (Jaiswal *et al.*, 2012). Although modern drugs have already been used intensively, sometimes

these drugs are unable to control hyperglycemia and eventually the diabetic patient's condition would be affected (Ablat *et al.*, 2014).

The purpose of this study was to analyze the antidiabetic effects of wood extracts as potentials anti-diabetic medicine. The wood is selected based on their high content of methanol extracts with a variety of phenolic compounds that may have the ability to inhibit α -glucosidase.

Phenolic compounds with specific structural features may bind enzymes by complex formation through hydrogen bonding or other mechanisms (Rahman *et al.*, 2017). Therefore, we evaluated the effect of phenolic extracts of wood extracts on the inhibition of α -glucosidase to test their potential antidiabetic effects and enhancement of the body's host immune system. The inhibitory effect of wood phenolic extract on α -glucosidase in *in vitro* assay is shown in Table 4.8.

Several previous reports have revealed that many plants species are used in traditional medicine for the management of diabetic complications showing α -glucosidase inhibition (Nkobole *et al.* 2011). For wider acceptability of the health benefits relevant to SPW, OPW, OW, QW, PW, and BW, the extract of these woods and its various solvent soluble fractions were evaluated for their antidiabetic activity by measuring α -glucosidase activity, and Tannic acid was applied as positive control.

The results showed that the SPW extracts exhibited the highest α -glucosidase inhibitory activity, higher than Tannic acid as well (Table 4.7). The percentage of inhibition values of wood extracts ranged from 20.03 to 90.35 % for soluble form. Insoluble-bound of wood extracts showed lower activity in terms of inhibition of α -glucosidase compared to the soluble extracts. Thus, an *in vitro* evaluation of wood extracts clearly depicts the inhibition of α -glucosidase enzyme responsible for high blood glucose level.

Wood extracts showed dual inhibiting potential against α -glucosidase enzymes (Table 4.8). SPW extract showed the highest inhibition of $90.35 \pm 19.73\%$ in soluble form and $45.92 \pm 9.09\%$ in the bound form on α -glucosidase, at a concentration of 10 μ L. α -Glucosidase is the important enzyme in the carbohydrate metabolism and as target for the therapeutics of diabetes, a major metabolic disorder in both developing and developed countries due to changes in people's lifestyle and dietary habits. Inhibition of α -glucosidase results in delayed carbohydrate digestion and glucose absorption with attenuation of postprandial hyperglycaemic excursions. Acarbose (the first dual inhibitor), miglitol, metformin and voglibose are commercially available enzyme inhibitors for type II diabetes. However, these drugs have been reported to cause various side effects such as abdominal distention, flatulence and possibly diarrhea (Ranilla *et al.*, 2008). The search for safe and effective inhibitors from natural sources are of emerging interest. Kumar *et al.* (2012) and Chairman *et al.* (2012) have examined the phenolics of wood in terms of inhibition of α glucosidase; wood extracts showed dual inhibitory potential against the target enzymes which might be due to the presence of specific phenolics.

Table 4.8. α -Glucosidase inhibitory activity (%) of soluble and insoluble-bound of woods^I

Sample	Soluble	Bound
Tannic acid	80.67±12.34 ^b	80.67±12.34 ^a
SPW	90.35± 19.73 ^a	45.92±9.09 ^b
OPW	85.80±14.01 ^b	38.67±7.69 ^b
OW	50.86±2.61 ^c	11.21±0.00 ^d
PW	26.96±6.69 ^d	8.07±0.46 ^d
QW	66.68±10.14 ^c	20.95±4.66 ^c
BW	20.03±5.74 ^d	4.45±0.003 ^d

^IData represent mean values ± standard deviation (n = 3). Values followed by the same letters within a column are not significantly different (p > 0.05)., n is the number of observed samples.

4.4.4. MALDI-TOF-MS analysis of structural phenolics of wood extracts

The structural identification of the phenolics extracted from woods that selected in this study, was done by using MALDI-Matrix Assisted Laser/Desorption Ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in conjunction with tandem mass spectrometry using high energy collision dissociation CID-TOF/TOF-MS/MS (MS/MS in-space) instrument. Table (4.9) presented the major protonated molecules of the phenolic compounds that we identified from the six different woods (SPW, OPW, OW, QW, and BW).

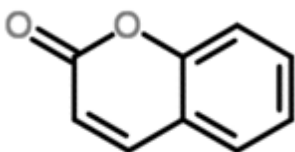
Table 4.9. Mass/Spectral characteristics of phenolics and other natural compounds identified from woods

Compound	MW	[M+H] (m/z)	Identification	Wood Identified
Hydroxycinnamic acids				
1	146.14	[C ₉ H ₆ O ₂ +H]	Coumarin	OPW,OW, QW, BW
2	208.21	[C ₁₁ H ₁₂ O ₄ +H]	Sinapaldehyde	OPW, SPW
3	178.18	[C ₁₀ H ₁₀ O ₃ +H]	Coniferilaldehyde	SPW, OPW, QW
4	176.17	[C ₉ H ₆ O ₄ +H]	6, 7-Dihydroxycoumarin	OW, QW, BW
5	196.16	[C ₉ H ₈ O ₅ +H]	Hydroxycaffeic acid	OW, QW, BW
6	326.29	[C ₁₅ H ₁₈ O ₈ +H]	<i>p</i> -Coumaric acid 4-o-glucoside	SPW, OPW, QW, PW
7	327.48	[C ₂₂ H ₃₁ O ₂ +H]	Unknown compound	SPW, OPW, QW, PW
Hydroxybenzoic acids				
8	154.12	[C ₇ H ₆ O ₄ +H]	Protocatechuic acid	SPW, OPW, QW, PW
9	198.17	[C ₉ H ₁₀ O ₅ +H]	Syringic acid	SPW, OPW, QW, PW
10	182.17	[C ₉ H ₁₀ O ₄ +H]	Homovanillic acid	SPW, OPW, QW, PW
11	312.37	[C ₁₉ H ₂₀ O ₄ +H]	Unknown compound	SPW, OPW, QW, PW
Flavonoids				
12	290.26	[C ₁₅ H ₁₄ O ₆ +H]	(+)-Catechin	SPW, OPW, QW, PW, BW
13	306.27	[C ₁₅ H ₁₄ O ₇ +H]	(+)-Gallocatechin	OW, OPW, QW, PW, BW
14	286.24	[C ₁₅ H ₁₀ O ₆ +H]	3,5,7,4'-Tetrahydroxyflavone	OW, QW, PW, BW
15	316.26	[C ₁₆ H ₁₂ O ₇ +H]	Rhamnetin	QW, PW, BW
16	272.25	[C ₁₅ H ₁₀ O ₅ +H]	3',4',7-Trihydroxyisoflavanone	OW, QW, PW, BW
17	314.28	[C ₁₇ H ₁₄ O ₆ +H]	5',7'-Dihydroxy-3',4'-dimethoxyisoflavone	OW, QW, SPW, BW
18	110.11	[C ₆ H ₆ O ₂ +H]	Catechol	OW, QW, PW, BW
Lignans				
19	358.38	[C ₂₀ H ₂₂ O ₆ +H]	Pinoresinol	OW, OPW, PW, BW

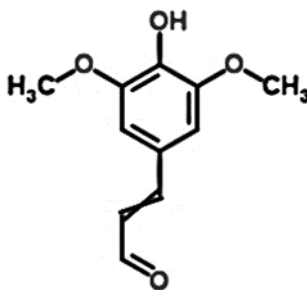
4.4.5. Structure–activity relationships (SAR) of phenolics from wood varieties

The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons, or chelate metal cations (Balasundram *et al.*, 2006). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure–activity relationship (SAR). In the case of phenolic acids/ flavonoids, for example, the antioxidant activity depends on the number and position of the hydroxyl groups in relation to the carboxyl functional group (Kerry *et al.*, 1999). Hydroxycinnamic acids exhibit higher antioxidant activity compared to the corresponding hydroxybenzoic acids (Andreasen *et al.*, 2001). Wood varieties, which have been studied in this research (SPW, OPW, OW, QW, PW, and BW), contain significant amounts of phenolic compounds, particularly SPW, OPW, OW, and QW, as presented in Table 4.9.

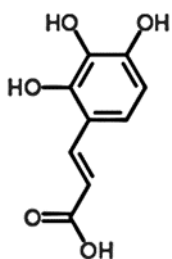
The presence of their phenolics may be considered as being desired for different applications due to their potential antioxidant properties as described for numerous phenolic compounds (Figure 4.5 (A) and (B)).



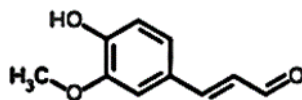
Coumarin



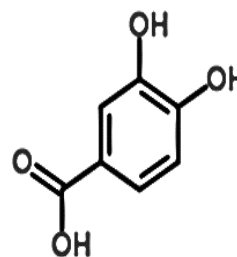
Sinapyl aldehyde



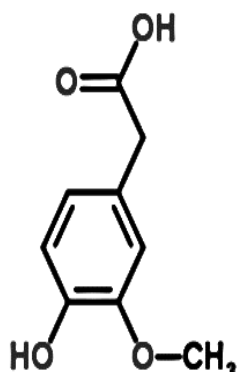
Hydroxycaffeic acid



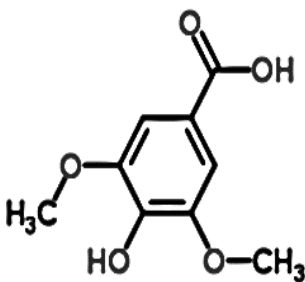
Coniferyl aldehyde



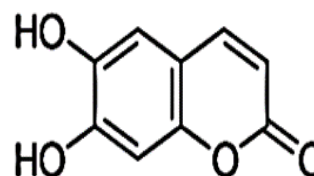
Protocatechuic acid



Homovanillic acid



Syringic acid



6,7-Dihydroxycoumarin

Figure 4.5 (A). Natural phenolics compounds identified from SPW, OPW, OW, QW, PW, and BW woods

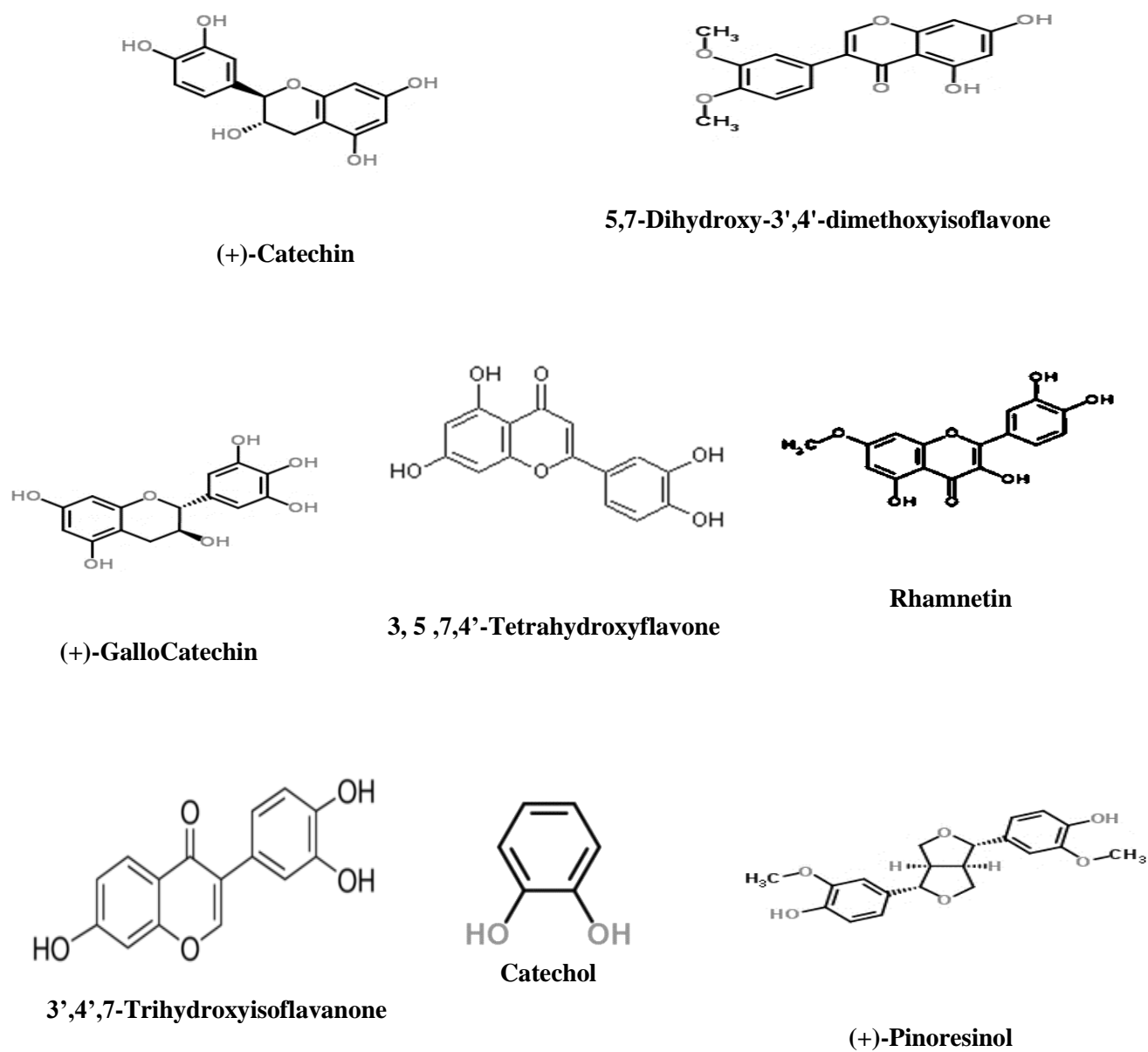


Figure 4.5 (B). Natural phenolics compounds identified from SPW, OPW, OW, QW, PW, and BW woods

4.4.6. Phenolic compounds from woods as natural sources of antioxidants

Plants as by-product source are rich sources of bioactive compounds, including phenolic compounds (Schieber *et al.*, 2001). The availability of phenolic compounds from agricultural and industrial residues, their extraction and antioxidant activity, have been reviewed by Moure *et al.* (2001). Phenolic compounds with antioxidant activity have been identified in several agricultural by-products, such as rice hulls (Ramarathnam *et al.*, 1989), buckwheat hulls (Watanabe *et al.*, 1997) and almond hulls (Takeoka *et al.*, 2002). The by-products of the olive industry have attracted considerable interest as a source of phenolic compounds, with much attention focused on the olive mill wastes (Balasundram *et al.*, 2006).

Maier *et al.* (2015) recorded that phenolic acids were present in *Quillaja* wood extracts; their occurrence might be useful for the detection of *Quillaja* extracts in foods, cosmetics, and pharmaceutical formulations. Royer *et al.* (2011) highlighted that the phenolics obtained from various tissues of red maple (branches, barks, and twigs) considered as forest industry residues, can be advantageously utilised as sources of potent antioxidants. Hydroxybenzoic acids (benzoic, salicylic, 4-hydroxybenzoic, procatechuic, gallic, syringic and vanillic acids), hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic acid, and (+)-catechin) have been extracted from *U. tomentosa*' leaves, stem, bark, and wood, and found to be as a suitable raw material for obtaining phenolic- and alkaloid-rich extracts of potential interest (Hoyos *et al.*, 2015). The lignin from hardwood and softwood could be attributed to its excellent adhesion ability on the metal surface and superior lubrication film strength (Mu *et al.*, 2018). Oak polyphenols possess various activities, such as anticancer, antioxidant, antidiabetic, antihypertensive, antimicrobial properties, and free radical scavenging capacity (Madrera *et al.*, 2010; Setzer, 2011). Almi *et al.* (2015) showed that the date palm wood is a good candidate for realization of natural composites with

several applications: building materials, automobile and furniture industries, while Nasser *et al.* (2016) investigated that the date palm tree could be considered more suitable source for energy production.

The results of our study showed that SPW, OPW, OW, QW, PW, and BW wood and bark extracts can constitute potential sources of natural antioxidant agents rich in polyphenols (Table 4.9). SPW and OPW particularly have not been studied for their soluble (SP) and insoluble-bound phenolics (IPB), and to our knowledge, this is the first study that presented the composition of both group of phenolics, to give more information of their antioxidant activities. In addition, extracted phenolics methods (Methanol/ acetone/ water) of woods that selected in this study exhibited in various fields, in foods, cosmetics, pharmaceuticals, as an environmentally friendly and low-cost solvent. Royer *et al.* (2011) which already find applications as additives in food, nutraceuticals, and cosmetic products to prevent damage provoked by oxidative stress in the human organism. We anticipated a real potential of date palm wood extracts following the integration wood industry of by-products, which will be thus transformed into radical scavenging and antioxidant capacities of the future.

CHAPTER 5

MALDI-MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY ANALYSIS OF A SERIES OF PROTONATED LIGNIN MOLECULES EXTRACTED FROM DATE PALM WOOD

This chapter represents a manuscript sent to the journal of Rapid Communication in Mass Spectrometry and as such contains an introduction to lignin structure and mass spectrometry and tandem mass spectrometry analyses. This manuscript is entitled as follows:

“Top-down lignomic maldi-tof-tandem mass spectrometry analysis of lignin oligomers extracted from Saudi date palm wood (SDPW)”. Tasahil Albishi, Abanoub Mikhael, Fereidoon Shahidi, Travis Fridgen, Joseph Banoub^a

5.1 LIGNIN INTRODUCTION

For almost a century, the structure of lignin was designated as a complex polymer composed of irregular branched units (Kosyakov *et al.*, 2016). Lignin is the second most abundant biopolymer in nature after cellulose; it is a product of enzymatic oxidative polymerization of three monomeric aromatic compounds (monolignols), namely coniferyl (H), sinapic (S), and *p*-coumaric (G) alcohols (Figure 5.1). It is found in all vascular plants, mostly between the cells, as well as within the cells and in the vegetable cell walls (CWs) (Vanholme *et al.* 2010). Lignin is relatively hydrophobic and aromatic in nature (Figure 2.4) and consists of several types of substructures, which possess the same phenylpropanoid skeleton, but differ, in the degree of oxygen substitution on the phenyl ring. The monolignol H-structure consists of a 4-hydroxyphenyl ring. The monolignol guaiacyl G-structure contains one hydroxyl and one methoxy group, whereas the monolignol syringyl structure contains two methoxy groups and one hydroxyl group as presented in Figure 5.1 (Dolk, *et al.*, 1986).

The polyphenolic structure of lignin imposes to the woody biomass resistance to biological and chemical degradation. This resistance is mainly due to the lignins hydrophobic nature and insolubility in aqueous systems, preventing access of degrading chemicals and organisms (Doherty *et al.* 2011).

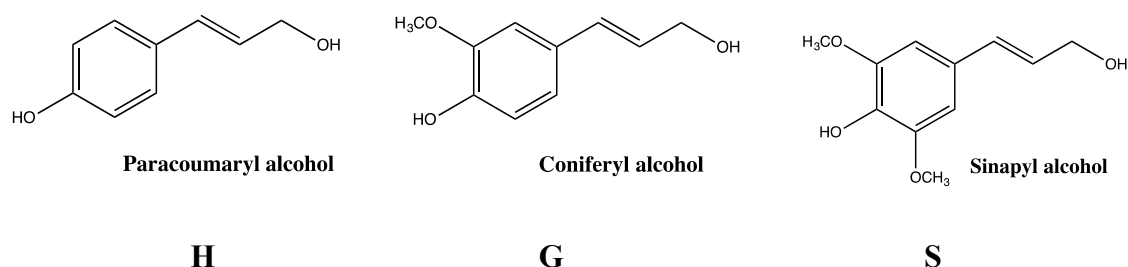


Figure 5.1. The three monomeric building units of lignin in nature

The H, G, and S constituent units are linked covalently, forming ether, ester and carbon–carbon bonds, which repeat in an apparently random manner and provide great complexity (Glasser, 1980). In general, the site of attachment of the covalent linkages can vary between the two units. It is generally accepted that lignins are composed of H, G and S units attached by β –O–4', β –5', β – β ', β –1', 5–5' and 5–O–4' linkages that are relatively resistant towards degradation (Campbell and Sederoff, 1996). These are designated according to the atomic centres in the radicals that are coupled together during the final step of lignin biosynthesis (Glasser, 1980). The equivalent substructures in the presumed overall structure of the biopolymer consist of alkyl–aryl ethers, phenylcoumarans, resinols, dibenzodioxocins, biphenyls, tetrahydrofuran-spiro-cyclohexadienones and diaryl ethers as showed in Figure 5.2 (Banoub and Delmas, 2003).

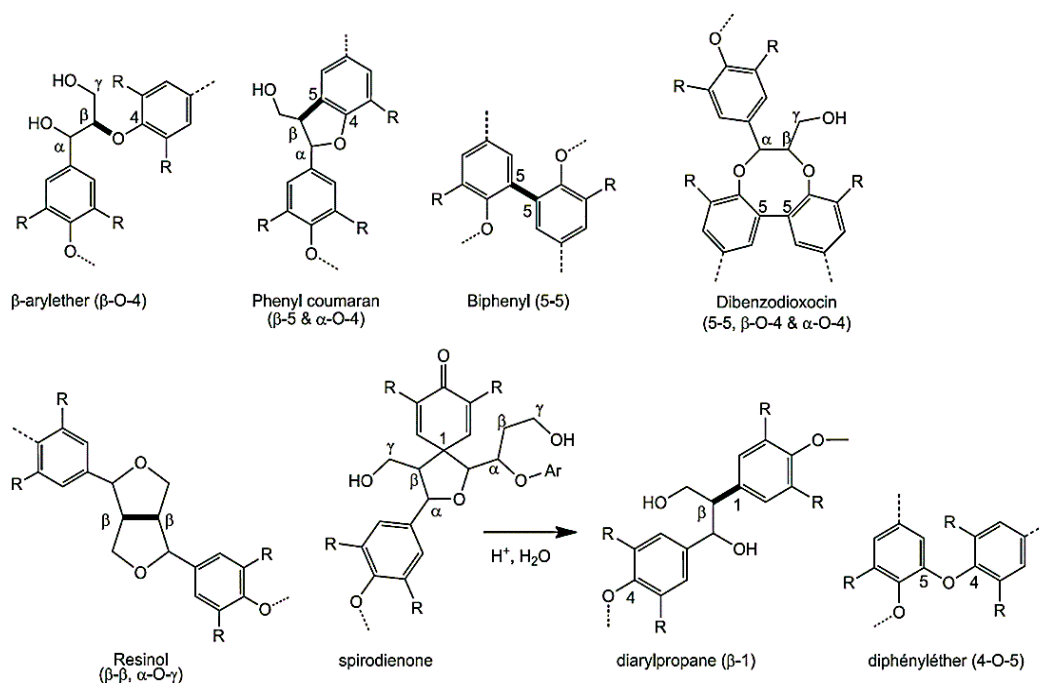


Figure 5.2. Main linkages in native lignins (R = H in *p*-hydroxyphenyl units or OCH₃ in guaiacyl and syringyl units). Reproduced with permission from C. Lapierre (2010). Copyright 2010 by Taylor and Francis Group, LLC.

In recent years, it was shown that the chemical structure of the various lignin molecules depends on the botanical origin and chemical composition of the vegetal fibres (Bauer *et al.* 1973). Several different chemicals, enzymatic and mechanical extraction methods have been proposed to be accountable for the major structural divergence that occurs after extraction and isolation of lignins (Bauer *et al.*, 1973; Banoub *et al.* 2015). As a result, it appears that the only logical step to determine the natural structure of lignin is to isolate it from the vegetal matrix, without causing any structural change (Delgado *et al.* 2003). Therefore, preparing pure samples of unchanged lignin is not an easy endeavor with the consequence that structural determination of

lignin is perhaps more challenging than with other biopolymers (Tronchet *et al.* 2010; Freudenberg, 1959; Brunow, 2001).

Generally, it is accepted that lignin exists as a cross-linked amorphous macromolecular material composed of phenylpropanoid lignol monomers. In softwood lignin, the G structure is dominant (Lapierre, 2010). Hardwood lignin normally contains a mixture of S and G lignols, with dominance of S. The H lignols predominate in lignin found in grasses (Wang *et al.* 2009). It was speculated that lignin may exist as one single molecule in its native environment and that its structure may never cease to grow in size (Croteau *et al.* 2000; Dolk *et al.* 1986). Accordingly, the chemical structure of lignin was described as a highly cross-linked and three-dimensional biopolymer. It was also suggested that the *in situ* molecular structure of lignin in the species of Japanese cedar (*Cryptomeria japonica*), camellia (*Camellia japonica*) and ginkgo (*Ginkgo biloba*) behaved much as if it was made up of linear macromolecules (Macha and Limbach, 2002; Chen, 1998). These newest views have been reported by many researchers (Figure 5.3) (Anterola and Lewis, 2002).

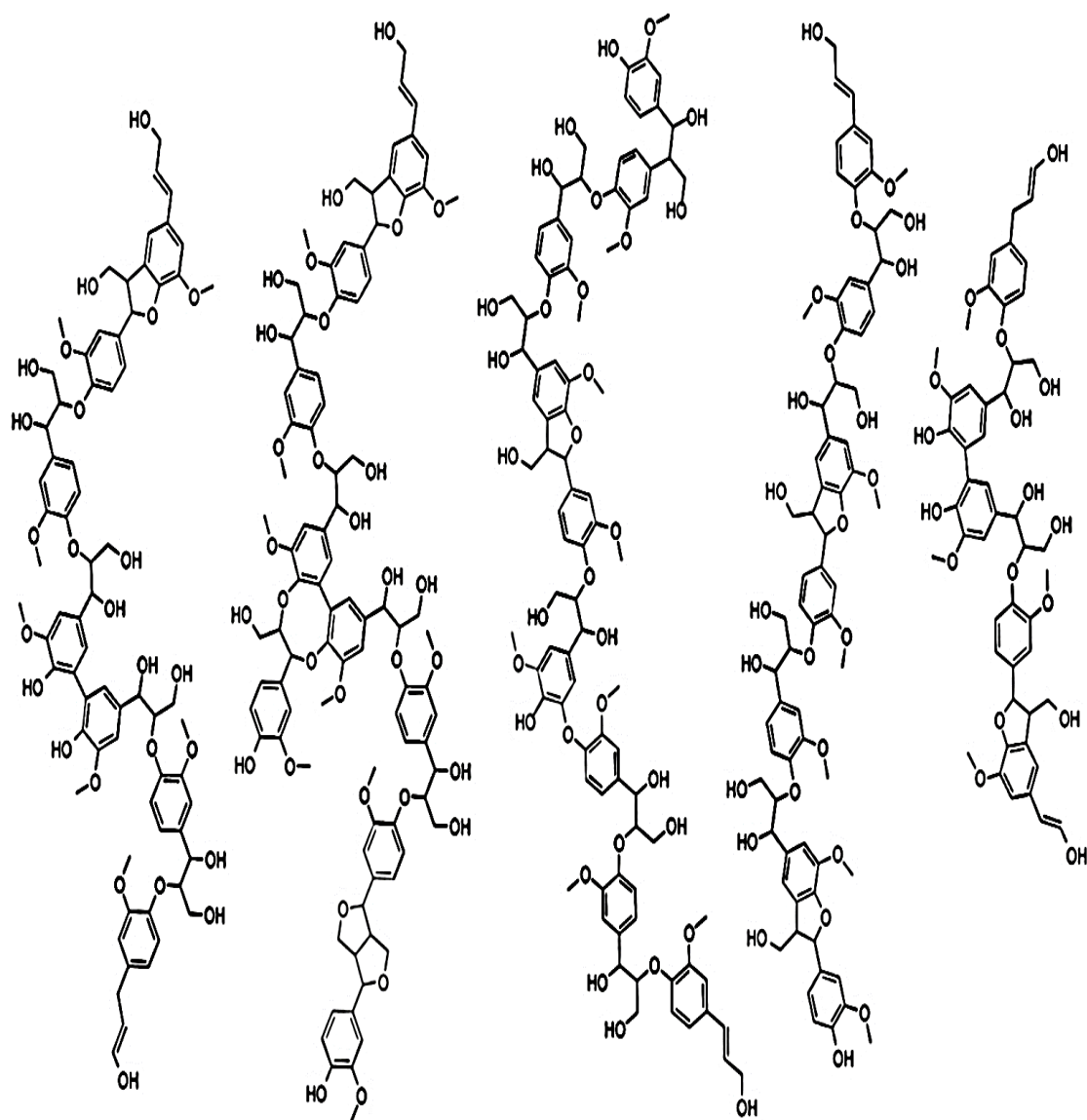


Figure 5.3. Schematic representation of softwood milled wood lignin. Reproduced with permission from C. Crestini *et al.* (2011). Copyright 2011 American Chemical Society.

Recently, two types of lignin analysis have been recognized. Usually, non-destructive analytical methods employing topochemical exploration are normally used (George *et al.*, 2011). For more than a century, lignin research has produced an enormous amount of experimental results that are to a large extent dissimilar and difficult to reproduce. The main reasons for these irregularities between experimental results have been attributed to the differences in the harsh extraction methods used. Presently, there is still much debate on whether any lignin extract adequately represents the native structure. For these reasons it was established that either strong acid or basic depolymerisation extraction method cause cleavage of ester and ether bonds, creating reactive species that could alternately react further to yield more complex and rearranged condensed lignin polymer/oligomer structures.

Consequently, the continued use of inadequate and outdated analytical methodology for characterizing both lignin polymer/oligomers (loved by conventional lignin chemists) further exacerbates the situation. Last, but not least, the mythical opinionated claim dictates that structural analysis of lignin should be based on pure samples (Gosselink, 2011).

It is well known that during lignin extraction, the β -O-4'dilignol linkage are the most normally and easily cleaved bond, while other dilignol linkages are chemically more difficult (Delmas, 1997; Banoub *et al.*, 2007). In addition, it is believed that the relative proportion of monomers dictates the relative abundance of inter-unit linkages present (Glasser, 1980). For example, lignins rich in G units contain more resistant β -5', β - β 'and β -O-4' linkages, whereas lignins enriched with S units are less cross-linked and less difficult to extract. That's why; lignin composition is classically described by the relative abundance and ratio of H, G and S units (Osono, 2002). As a result, our knowledge of lignin chemical structure is less precise than that of other natural and synthetic polymers (Vanholme *et al.*, 2010).

It is worth noting that lignin biosynthesis is regarded as a “Combinatory Process”. This theory postulates that oxidation enzymes produce lignol radicals, which combine to form dilignols, trilignols and oligolignols. The theory depends on nothing, except the chemical control that dictates the coupling and by association the oligomeric structures. This polymerization is initiated by the oxidative radical ionization of phenols, followed by combinatorial radical coupling. Decades of research are consistent with this model (Davin and Lewis, 2000) which still remains valid.

Currently, lignin is viewed as a promising commercial source of a wide range of aromatic compounds, alternative to fossil hydrocarbons. The worldwide annual production of lignin, as a side product of wood processing industries, exceeds 50 million tons (Davin and Lewis, 2005; Dolk *et al.*, 1986).

There is a growing interest by researchers in the structure of lignins of various plants, which, in spite of almost 100 years remain poorly understood. In this regard, mass spectrometric methods, can be used to unravel new sequencing method for the lignin oligomers and for interpreting the plant ‘lignome’.

Contrary to proteomics, glycomics and genomics, lignomics research, there is no explicit sequencing methods that can establish the primary structure of complex and simple lignin oligomers. Researchers are required to synthesize authentic compounds as standards to enable verification and comparison with the MS/MS scheme obtained by an unknown compound.

It is well acknowledged that lignin polymer and/or oligomer are one of the last biopolymers to defy attempts to determine their full sequences and structures. Nevertheless, shorter oligomers of lignin can be extremely useful in providing the blueprints on how the full lignin polymer is constructed. Limited structure elucidation of oligomers has been obtained following

dehydrogenative polymerization studies (Vanholme *et al.*, 2010). However, such experiments fall short of being able to deduce the structure of lignin because it can vary among cell types and individual cell wall (CW) layers.

5.2. MALDI-TOF-MS

MALDI (matrix-assisted laser desorption/ionization) is an elegant soft ionization method. MALDI is frequently coupled with a time-of-flight (TOF) mass analyzer (MALDI-TOF-MS). MALDI is undeniably a powerful “soft” ionization method suitable for almost all analytes extending from higher molecular masses such large and/or labile molecules, lipids, proteins, oligonucleotides, lignins, and synthetic polymers to small molecular weight compounds (Tanaka *et al.*, 1988; Schwamborn and Caprioli, 2010). In MALDI the analytes can either accept a proton or lose a proton by interaction with the MALDI matrix (- ion mode and + ion modes).

For the preparation of the MALDI plate, the samples are intimately mixed with the matrix and dried on a target (plate). The target is then loaded into the mass spectrometer where the sample/matrix is irradiated by the laser. This process transfers both energy and charge to the sample molecules; for example, in negative ion mode, the analyte molecules are transferred in the gas phase as deprotonated molecules. Ions are accelerated out of the source region by an electric field (20-30 kV) and fly down the flight tube which is typically about 1 m long. At the end of the flight tube, a detector records both the intensity of the ion current and the time of flight. The MALDI process, especially the ionization step itself, is in contrast to the impressive range of applications far less understood, although the desorption/ionization process has been studied in detail (Banoub, 2014). Based on the matrix and analyte ion velocities the formation of a gas jet carrying the analyte is plausible.

Contrary to common views, MALDI can be applied to very low mass analytes and provides accurate MW assignments. This makes it possible to assign elemental compositions (Bucknall *et al.*, 2010). Synthetic polymer characterization is a challenge exercise, but MALDI offers a powerful solution and its applications have been comprehensively reviewed by Weidner and Trimpin (2010).

In this chapter, structural identification of the extracted phenolics and the structural elucidation of series of lignin bio oligomers which were extracted by “La Compagnie Industrielle de la Matiere Végétale” (CIMV) solvolysis technique from the Saudi seedling tree are discussed. The CIMV was chosen as this technique appears to be the optimum technique for lignin separation and this is due to its capabilities of being efficient and allowing the choice of simple organic solvents such as acetic acid-formic acid-water combination. The structural identification of the extracted phenolics and virgin released lignin (VRL) will be performed by MALDI-Assisted Laser/Desorption Ionization (MALDI)-MS in conjunction with tandem mass spectrometry.

5.3. Methods

5.3.1. Samples

The samples date palm wood (*Phoenix dactylifera*) examined were collected manually from the Salman Alfarsi Garden, Almadinah, Saudi Arabia. All the samples were frozen and dried for 7 days at -48°C and 30×10^{-3} mbar (Freezone 6, model 77530, Labconco Co., Kansas City, MO). The dried samples were then grounded, vacuum packed and stored in a freezer at -20°C.

5.3.2. Lignin oligomers solation

The Saudi Date Palm Wood (SDPW) lignin was extracted using the CIMV procedure which selectively separates the cellulose, hemicellulose and lignin, and allows the destructing of the

vegetable matter at atmospheric pressure (Delmas and Avignon, 1997; Lam *et al.*, 2001). The catalyst-solvent system used was a mixture of formic acid/acetic acid/water (30/50/20) which produced after precipitation with water and filtered the Saudi Date Palm Wood (SDPW) lignin. Approximately 0.1mg of the purified lignin was dissolved in 1mL dioxan- methanol-chloroform (1:1:1, v/v/v) for MS analysis.

5.3.3. MALDI-TOF-MS analysis

The MALDI time-of-flight TOF/TOF mass spectrometer (MALDI 4700, Applied Bioscience) was used in this experiment for the analysis of the Saudi Date Palm Wood (SDPW) lignin. In this analysis, 1 mg of the lignin sample was dissolved in 1 mL of the dioxan/ methanol/chloroform (1:1:1) and 2,5- dihydroxy benzoic acid (DHB) was used as matrix for the analysis. The MS data was acquired in the mass range 100 to 2000 m/z in the positive ion mode. The mass spectra instrument was equipped with Nd: YAG 200-Hz laser. The accelerating potential was 25KV. The MALDI plate was prepared by spotting 1 μ L of a 20mg/ml solution of DHB (dissolved in acetone, 0.1% trifluoroacetic acid) and then dried at room temperature (the use of acetone allows a good homogeneity of the matrix in the spot). Then, an aliquot of 1 μ L of sample was spotted on the top of the dried matrix and allowed to dry before the MALDI-TOF-MS experiments. For MS analysis, mass spectra were the sum of 400 laser shots and acquired in reflectron mode. For high-energy collision-induced dissociation (CID)-MS/MS analysis, mass spectra were the sum of 600 laser shots, a collision energy of 1 keV, nitrogen as the collision gas to induce high energy CID-fragmentation. The following standards were used to calibrate the mass spectrometer: des-Arg1-Bradykinin, [C₄₄H₆₁N₁₁O₁₀], M.Wt. 904.0245 from Enzo Life sciences, Inc., Farmingdale, NY 11735, USA; Angiotensin 1, [C₆₂H₈₉N₁₇O₁₄], M.Wt. 1296.4779, from Tocris Bioscience, 614

McKinley Place N.E., Minneapolis, Minnesota 55413, USA. The differences for all CID-MS/MS analyses, between the calculated m/z and the observed m/z were around 5-10 ppm.

5.4. Results and discussion

5.4.1. MALDI-TOF-MS analysis of the VRL oligomer extracted from SPW wood

The MALDI-TOF-MS analysis of the SPW (Figure 5.4 and 5.5; Scheme 5.1 and 5.2) showed a series of protonated molecules *inter alia* at 381.1541; 752.6363; 780.2611; 957.3162; 1039.3382; 1055.3281; 1201.4098 and 1217.4427 (Table 5.1). The generation of one dimensional MALDI-TOF-MS scan and various high energy dissociation tandem mass spectrometry (CID-MS/MS) analyses will provide series of protonated molecules and MS/MS diagnostic product ions, which will serve as a tool for obtaining high-quality mass spectra of VRL of seedling date palm wood (*Phoenix dactylifera*) suitable for structural studies of the analyte biopolymer. In this chapter, for the sake of brevity, few examples of the high CID-MS/MS of two precursor ions at m/z 381,1541 (1), 752.6363 (2), 957.3162 (3), and m/z 1217,4427 (4) will be discussed only.

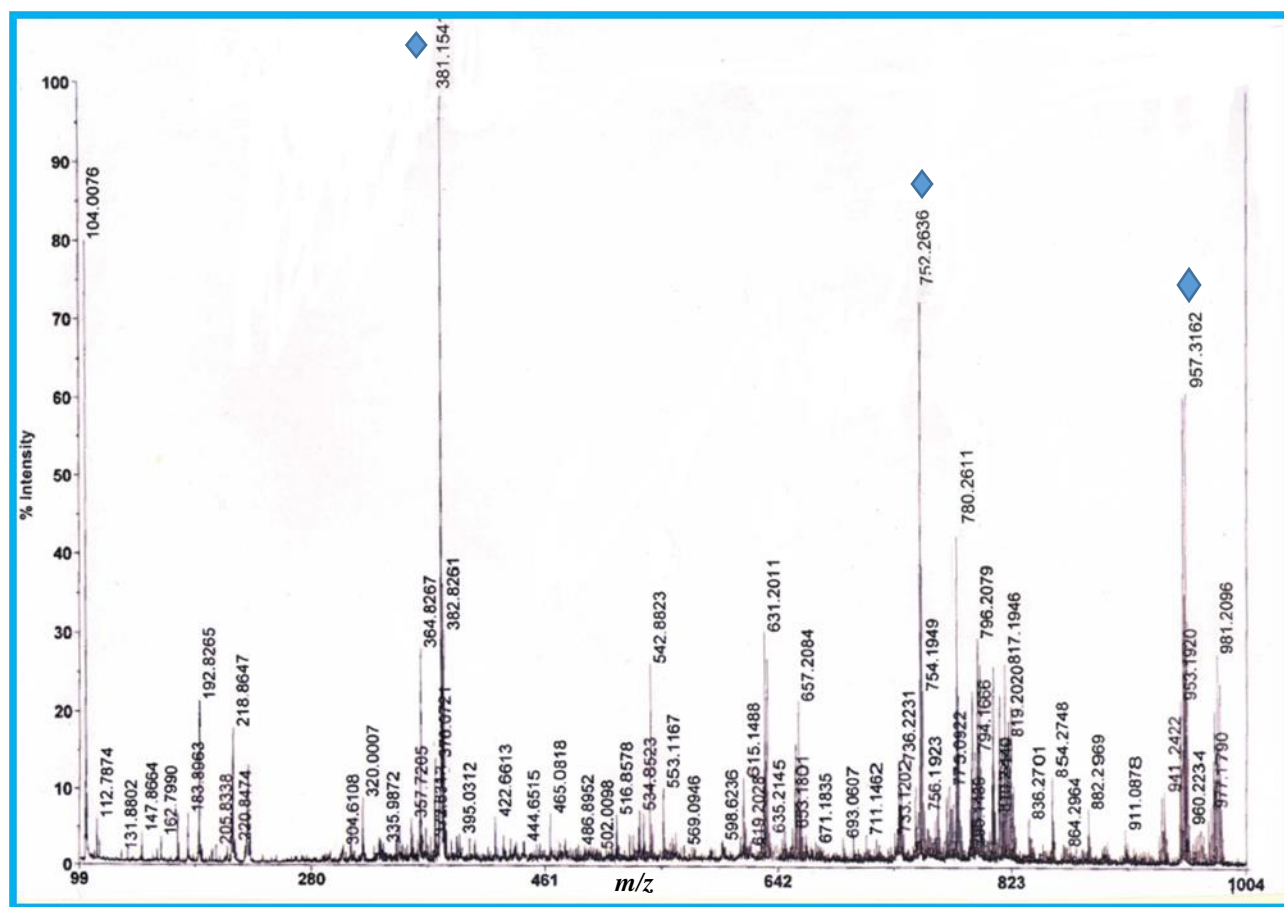


Figure 5.4. MALDI-TOF MS of phenolics and lignins from SPW (*Phoenix dactylifera*) wood

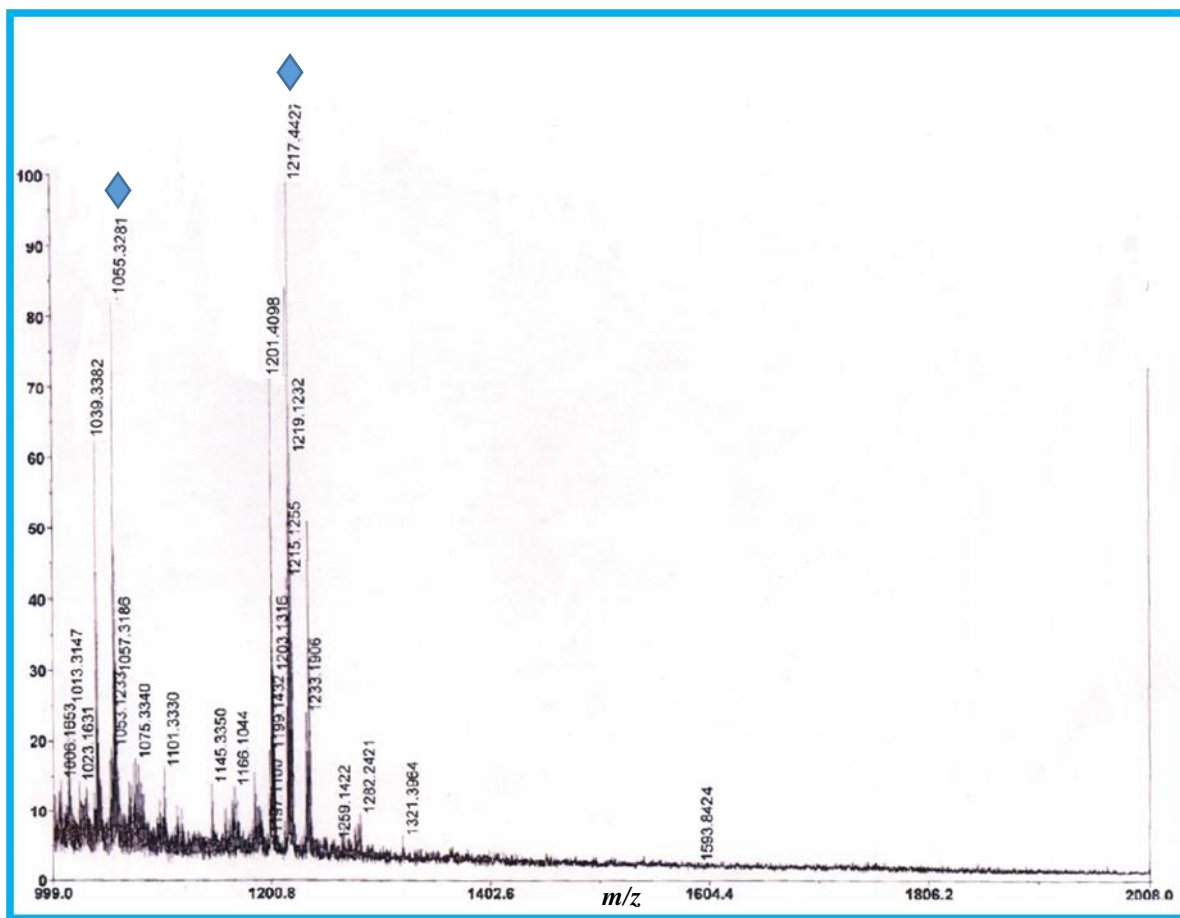


Figure 5.5. MALDI-TOF-MS of different lignins from SPW (*Phoenix dactylifera*)

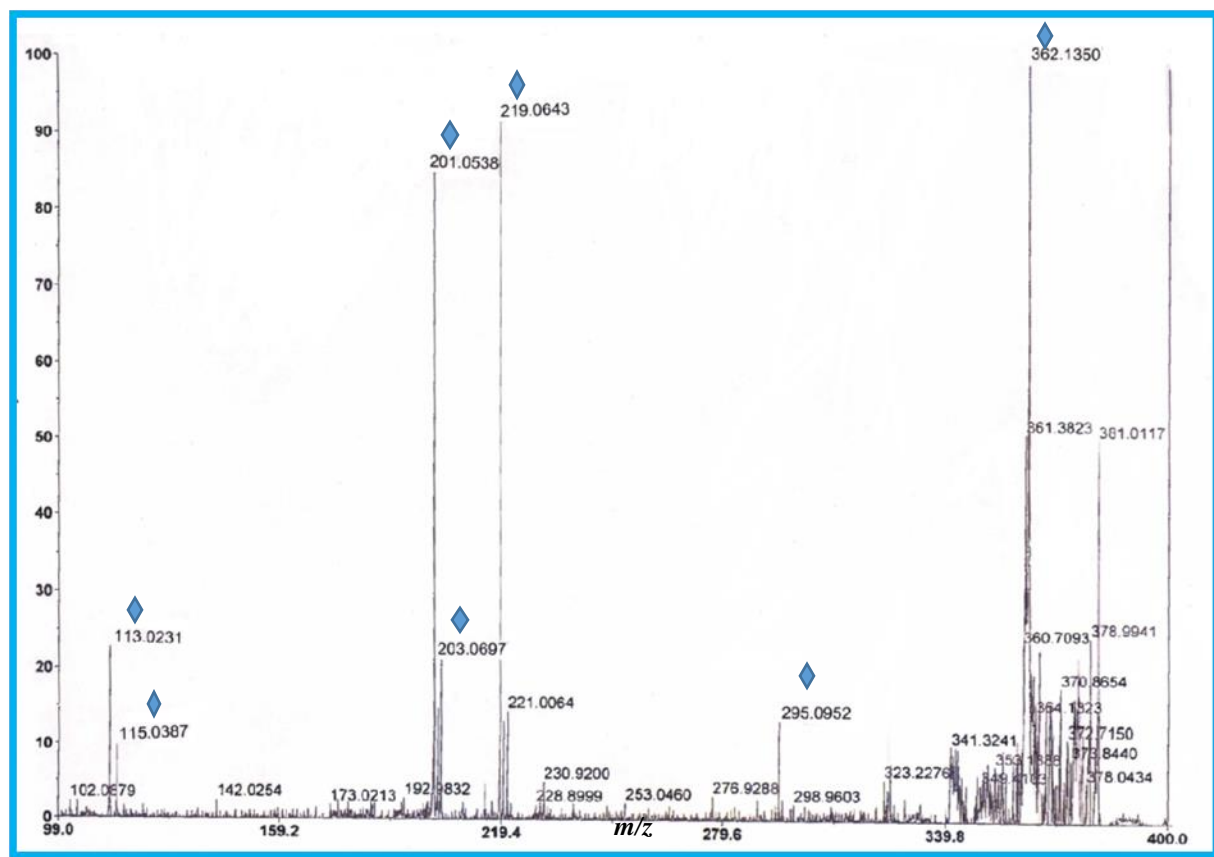


Figure 5.6. Tentative breakdown processes in the MS/MS of protonated at m/z 381.1532

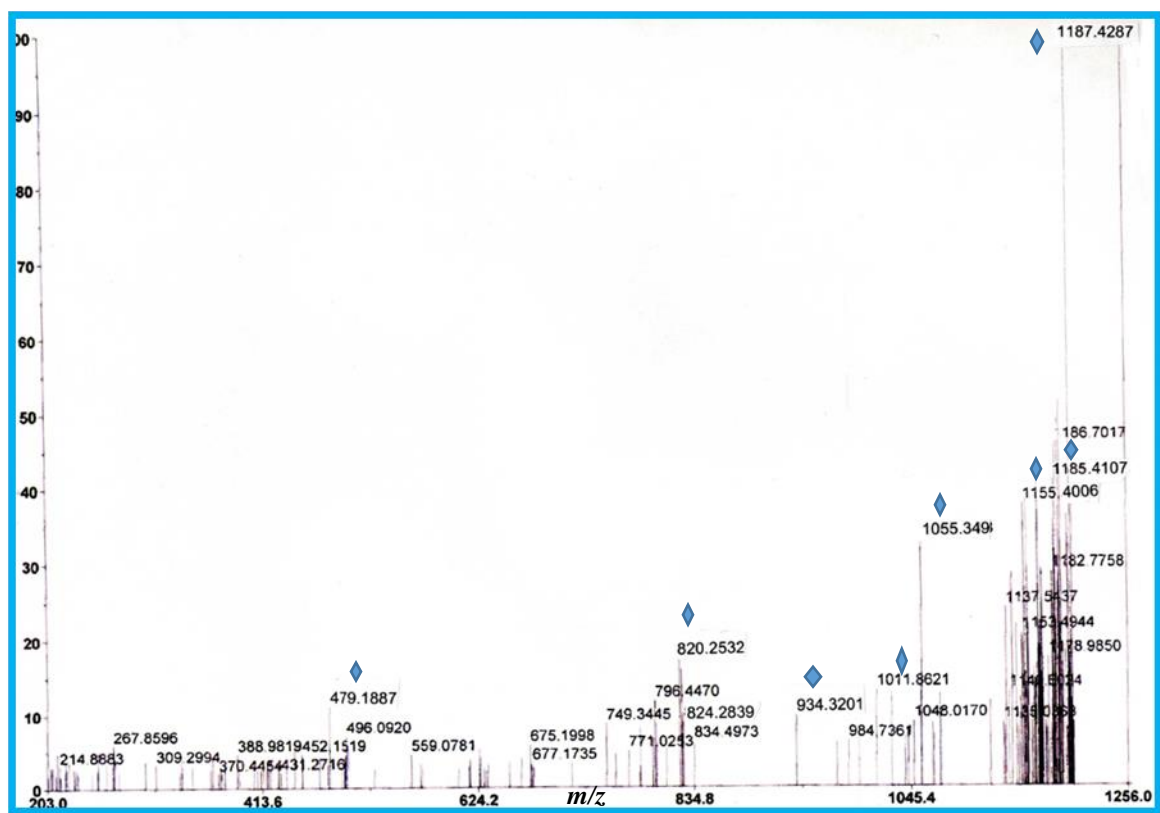
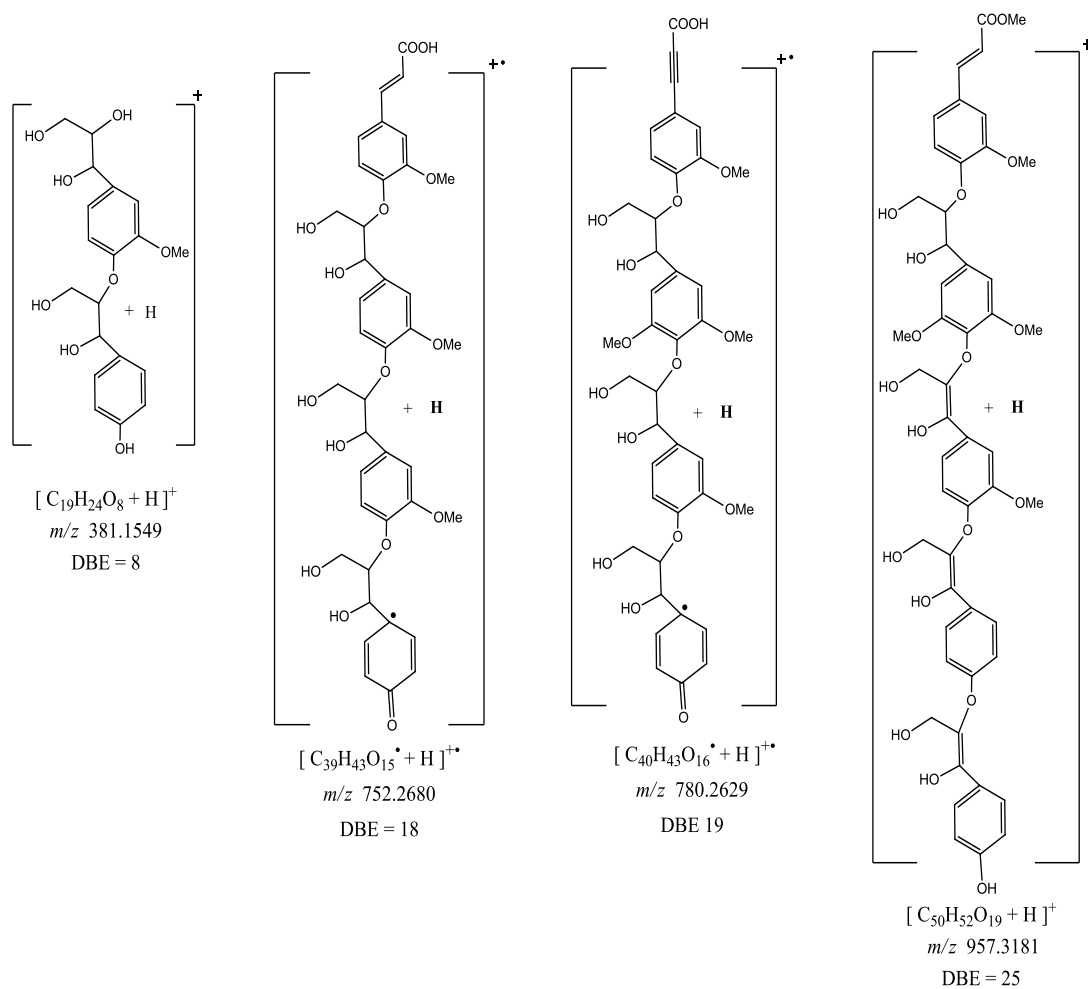
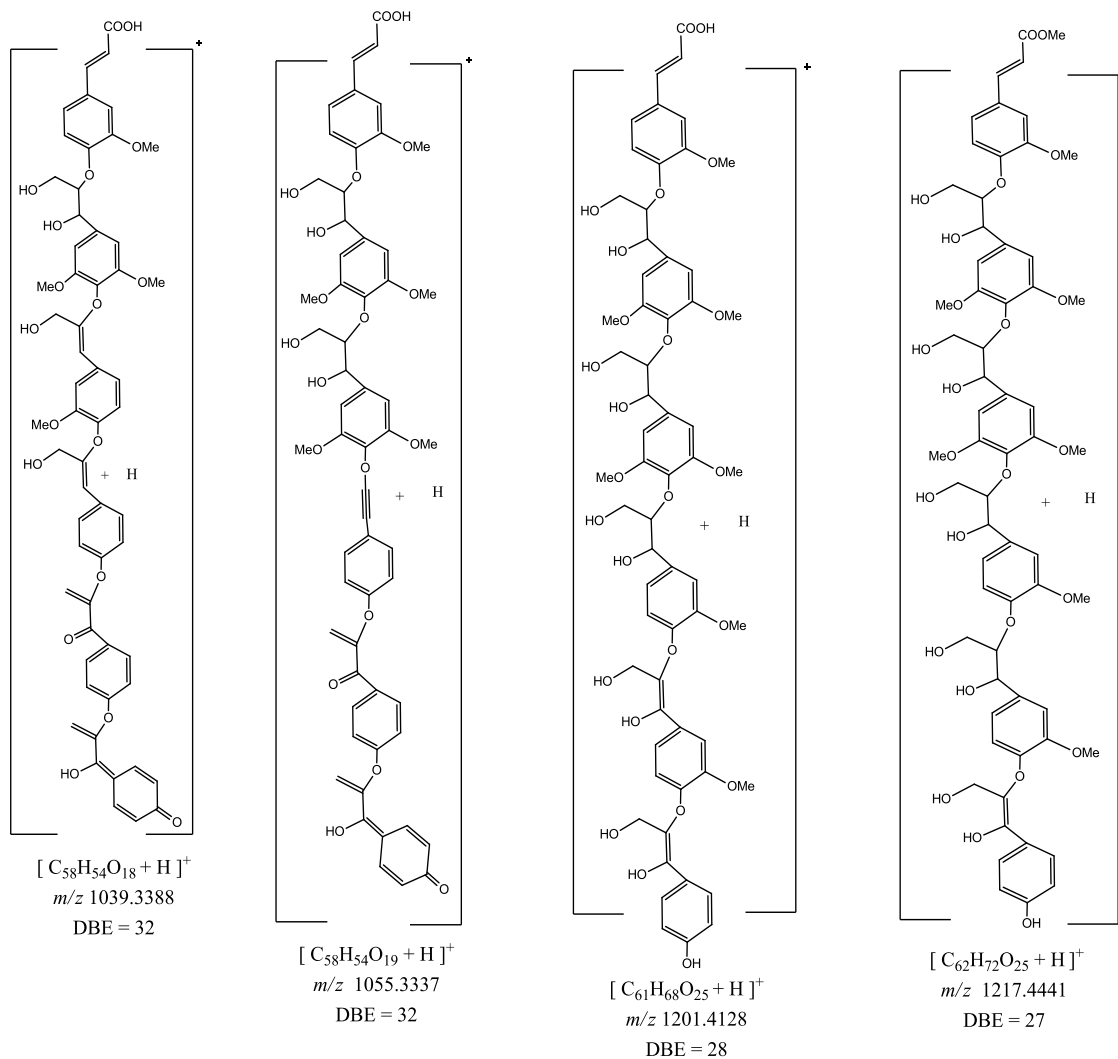


Figure 5.7. Tentative breakdown processes in the MS/MS of protonated at m/z 1217.4427



Scheme 5.1. Structures of the Lignin Oligomers identified for the Saudi date palm



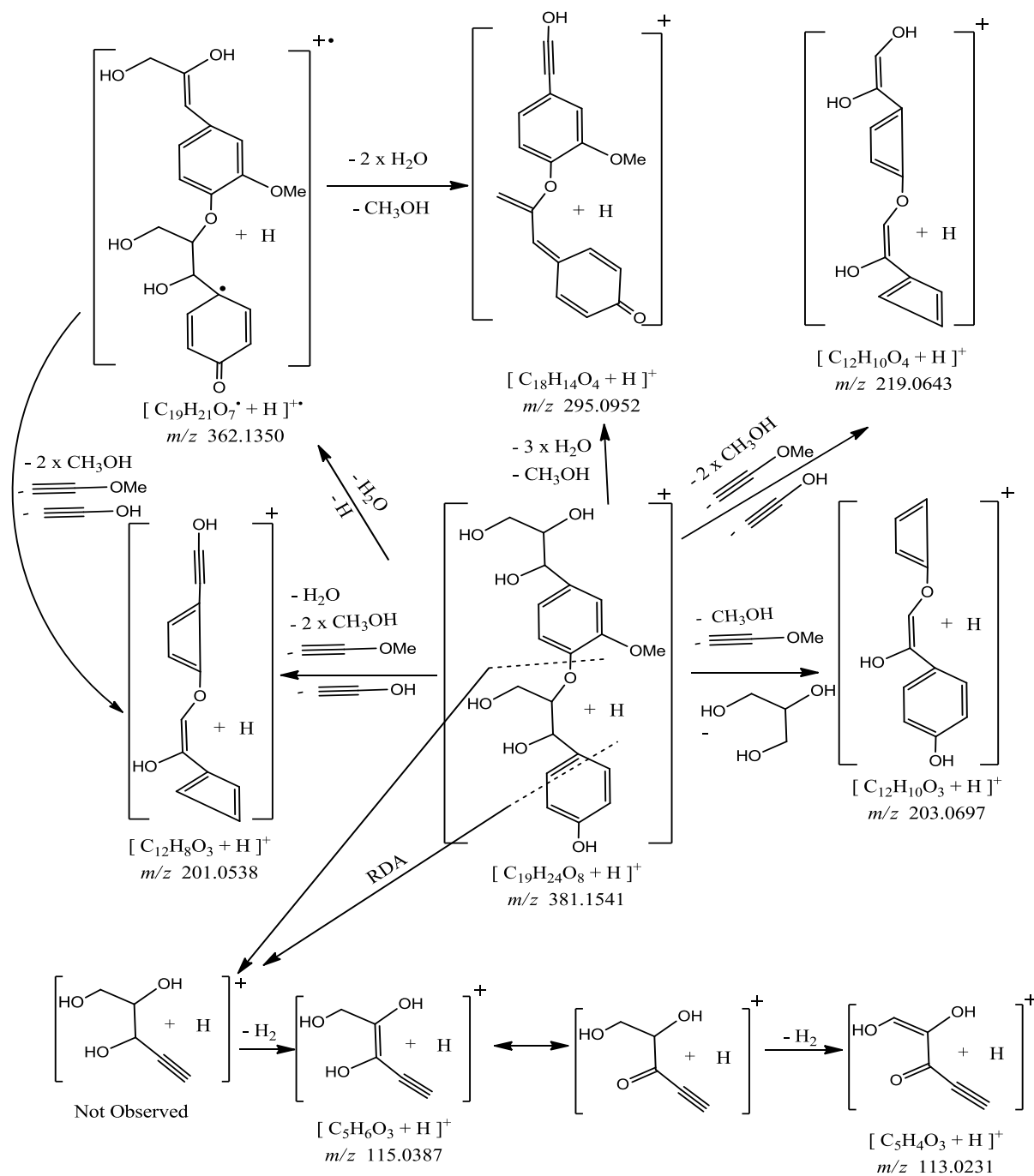
Scheme 5.2. Structures of the Lignin Oligomers identified for the Saudi Palm date at m/z 1039.3398; 1055.3281; 1201.4098 and 1217.4427

We begin with the identification of the chemical structure of the protonated molecules at m/z 381.1541 (scheme 5.1), having a DBE value 8, which was tentatively assigned to have the following chemical composition $[C_{19}H_{24}O_8 + H]^+$ and was attributed to H (8-O-4') G dilignol product having the structure (Scheme 5.3)

The product ion scan of m/z 381.1541 gave three major diagnostic product ions *inter alia* at m/z 201.0538; 219.0643; and 362.1350 which structures were defined respectively as $[C_{12}H_8O_3 + H]^+$; $[C_{12}H_{10}O_4 + H]^+$ and $[C_{19}H_{21}O_7 + H]^+$. The primary distonic protonated radical (Sack *et al.*, 1985; Holman *et al.*, 1986) product ion at m/z 362.1350, which possess an even m/z , was formed by the remote charge fragmentation mechanism (Wysocki and Ross, 1999; Gross, 2000) occurring by loss of a molecule of water and H radical from the precursor ion m/z 381.1541. The consecutive eliminations from the precursor ion at m/z 381.1541 of two methanol molecules along with retro-Diels Alder reaction (RDA) (Pandey *et al.*, 2014; Longo *et al.*, 2018) occurring in both H and G units (loss of hydroxy acetylene and methoxy acetylene) lead to the formation of the primary product ion at m/z 219.0643. Bear in mind that the formation of secondary product ion at m/z 201.0538 was created by the primary product ion m/z 362.1350. The high energy CID-MS/MS of the precursor ion m/z 381.1541 is shown in scheme (5.3).

Table 5.2. Identification of lignin Oligomers Peaks in Mass Spectrum of extracted from SDPW Wood using MALDI-TOF-MS

Compound	Molecular Formula	Calculated [M+H] ⁺ (m/z)	Observed (m/z)	Difference ppm	CID-MS/MS Product ions
1	[C ₁₉ H ₂₄ O ₈ +H] ⁺	381.1549	381.1541	-2.09	113.0231, 115.0387, 201.0538, 203.0697, 219.0643, 362.1350, 295.0952
2	[C ₃₉ H ₄₃ O ₁₅ +H] ⁺	752.2680	752.2636	-5.84	163.0384, 184.0724
2	[C ₃₉ H ₄₃ O ₁₅ +H] ⁺	752.2680	752.2636	-5.84	278.0773, 389.1209, 429.1887, 469.1831, 493.1833, 495.1979, 575.1887, 599.2092, 621.1929, 668.2049, 645.2655, 675.2389, 691.2709, 693.2871, 695.3022, 697.3181, 707.2667, 709.2467, 711.2601, 713.2766, 715.2911, 735.2591
3	[C ₄₀ H ₄₃ O ₁₆ +H] ⁺	780.2629	780.2611	-2.30	86.0362, 163.0388, 184.0724, 493.1821, 536.1429, 597.1931, 723.2236, 735.2613, 737.2399, 739.2545
4	[C ₅₀ H ₅₂ O ₁₉ +H] ⁺	957.3181	957.3162	-1.98	104.0620, 113.0229, 115.0389, 172.0725, 184.0727, 278.0773, 319.1159, 347.1114, 405.1518, 478.1801, 493.1465, 502.1368, 658.1801, 683.1655, 702.1683, 798.2101, 893.2615, 909.2912, 910.2633, 939.3014
5	[C ₅₈ H ₅₄ O ₁₈ +H] ⁺	1039.3388	1039.3382	-1.53	104.0619, 172.0722, 184.0719, 430.1392, 663.2196, 749.2343, 851.2856, 853.2593, 893.2962, 975.3177, 995.3432, 1009.3213, 1011.3371
6	[C ₅₈ H ₅₄ O ₁₉ +H] ⁺	1055.3337	1055.3281	-5.30	104.0619, 116.0466, 172.0721, 269.1004, 469.1673, 495.1832, 643.1979, 719.1866, 806.2685, 851.2649, 867.2398, 1011.3378, 1027.3319
7	[C ₆₁ H ₆₈ O ₂₅ +H] ⁺	1201.4128	1201.4098	-2.49	479.1253, 493.1678, 798.2684, 820.2523, 822.2679, 824.2849, 1055.3276, 1139.3692, 1155.4029, 1169.3803
8	[C ₆₂ H ₇₂ O ₂₅ +H] ⁺	1217.4441	1217.4427	-1.14	479.1887, 771.2819, 820.2532, 824.2839, 934.3201, 1055.349, 1155.4006, 1185.4107, 1187.4287

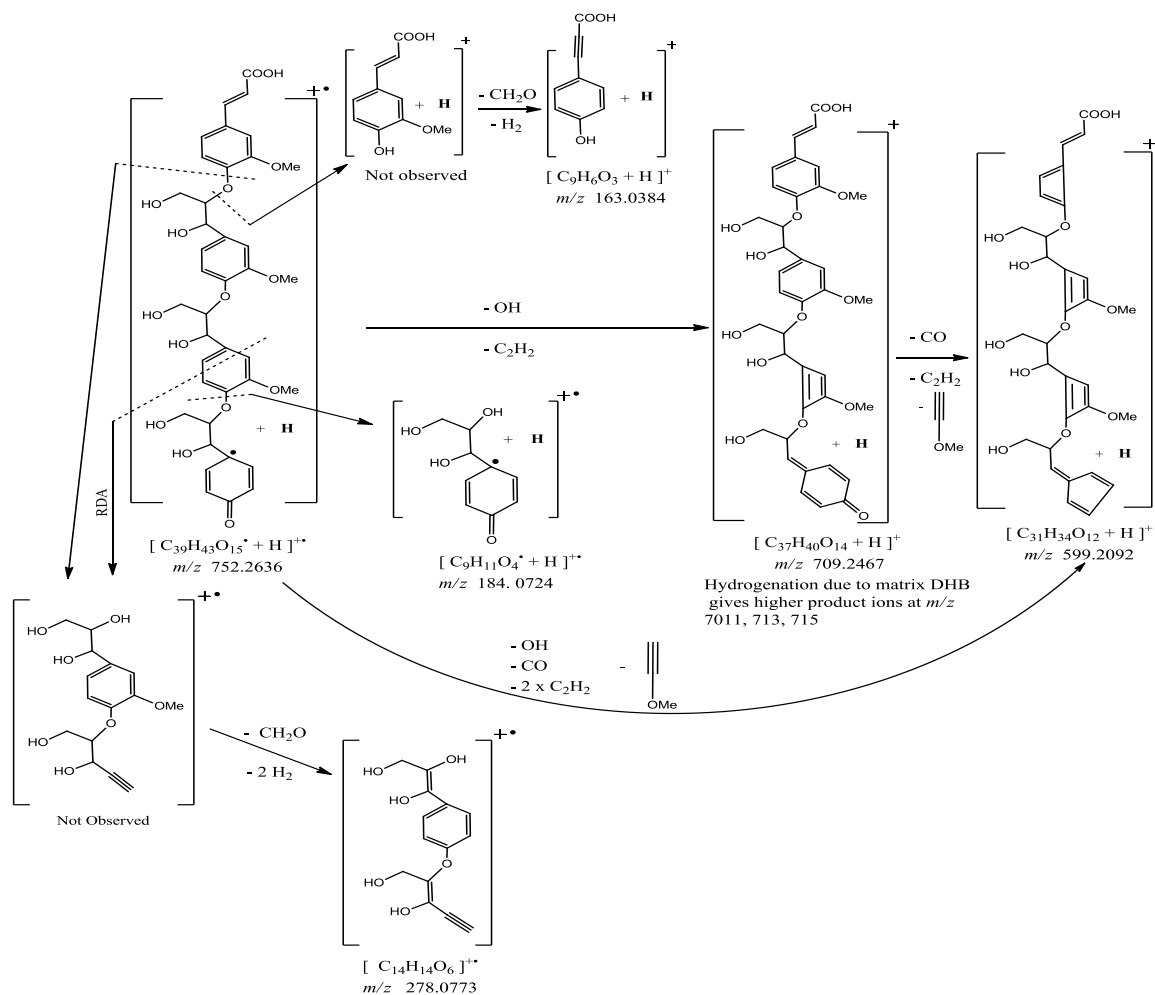


Scheme 5.3. High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 1 at m/z 381.1541

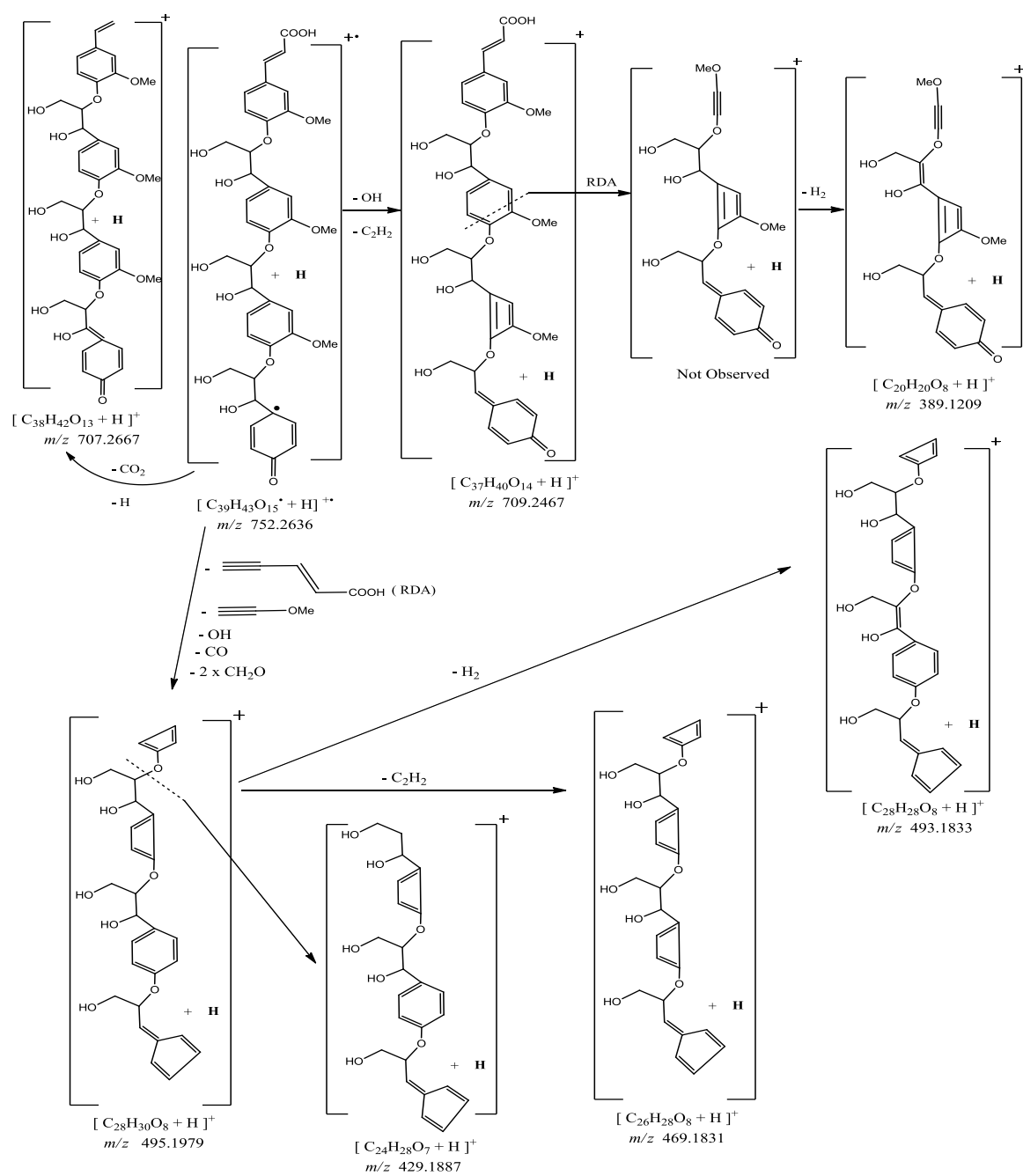
The protonated distonic radical molecule of (2) at m/z 752.2636, having a double bond equivalent (DBE) value 18, was assigned to the lignin tetramer H(8-O-4) G(8-O-4')G(8-O-4'')G (2) which had the molecular formula $[C_{39}H_{43}O_{15} + H]^+$. The product ion scan of the precursor ion (2) at m/z 752.2636 afforded the product ion at m/z 709.2467, which was formed by the loss of one ethyne molecule C_2H_2 eliminated by a retro-Diels Alder reaction (RDA) (Pandey *et al.*, 2014) and one hydroxyl group, and it was assigned as $[C_{37}H_{40}O_{14}+H]^+$. It is interesting to note the successive formation of the product ions at m/z 711.2601; 713.2766; and 715.2911. These latter product ions were assigned to have the following molecular formulae $[C_{37}H_{42}O_{14}+H]^+$, $[C_{37}H_{44}O_{14}+H]^+$ and $[C_{37}H_{46}O_{14}+H]^+$ and appeared to be formed by hydrogenation caused by the DHB matrix (Koomen and Russell, 2000). The product ion at m/z 709.2467 can lose a molecule of carbon monoxide and subjected to another two retro-Diels Alder (Pandey *et al.*, 2014; Longo *et al.*, 2018) reactions to afford the ion at m/z 599.2092. It is important to understand that the order of these eliminations could occur simultaneously or in a stepwise fashion and this was not studied further.

Moreover, the precursor distonic ion (Pandey *et al.*, 2014; Holman *et al.*, 1986) (2) can undergoes a concerted retro-Diels-Alder (RDA) reaction by cleavages of the C1-C2 and C5-C6 aromatic bonds of the fourth unit G and by cleavages of the C1-C2 and C3-C4 aromatic bonds of the third unit G, along with successive losses of carbon monoxide, hydroxyl group and two formaldehyde molecules to afford the product ion at m/z 495.1979. The exact order of these MS/MS gas-phase eliminations has not been established. This latter secondary product ion can either lose a molecule of hydrogen to afford the secondary product ion at m/z 493.1833 or it can be subjected to another retro-Diels-Alder (Pandey *et al.*, 2014; Longo *et al.*, 2018) reaction by loss of a molecule of ethyne to afford the ion at m/z 469.1831. In addition, the product ion at m/z 495.1979 can also be subjected to cleavage of the 8-O-4 bond between the upper two units to afford the secondary product ion at m/z 429.1887. Finally, the presence of carboxylic acid group was confirmed by the loss of CO_2 and H radical from the precursor ion at m/z 752.2636 to afford the product ion at m/z 707.2667 assigned as $[C_{38}H_{42}O_{13} + H]^+$. The high-energy CID-MS/MS

fragmentation patterns and lower m/z product ions of the precursor ion at m/z 752.2636 are shown in schemes (5.4 (A) and (B)).



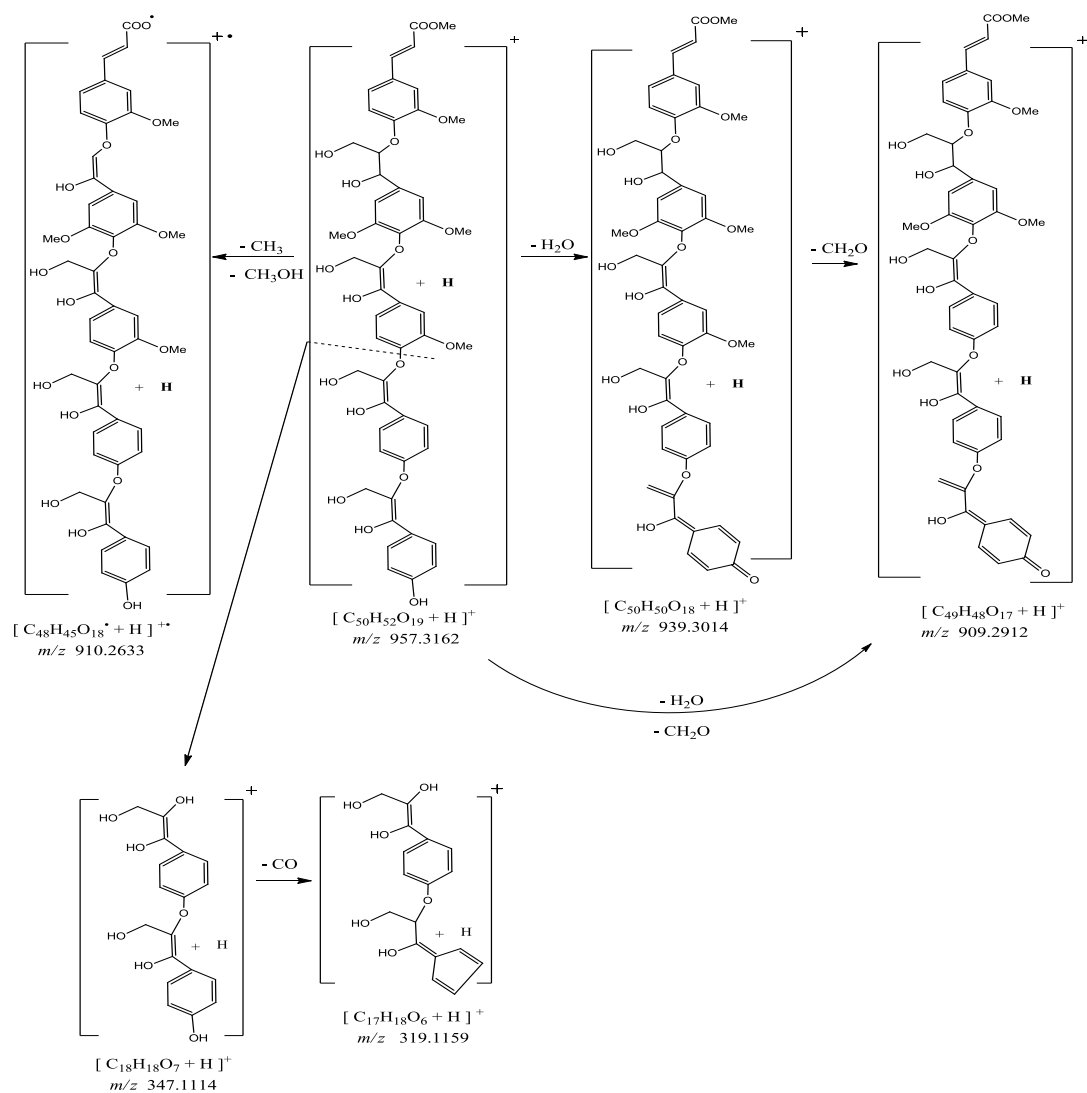
Scheme 5.4 (A). High-energy CID-MS/MS fragmentation pattern of the precursor dicationic cation 2 at m/z 752.2636



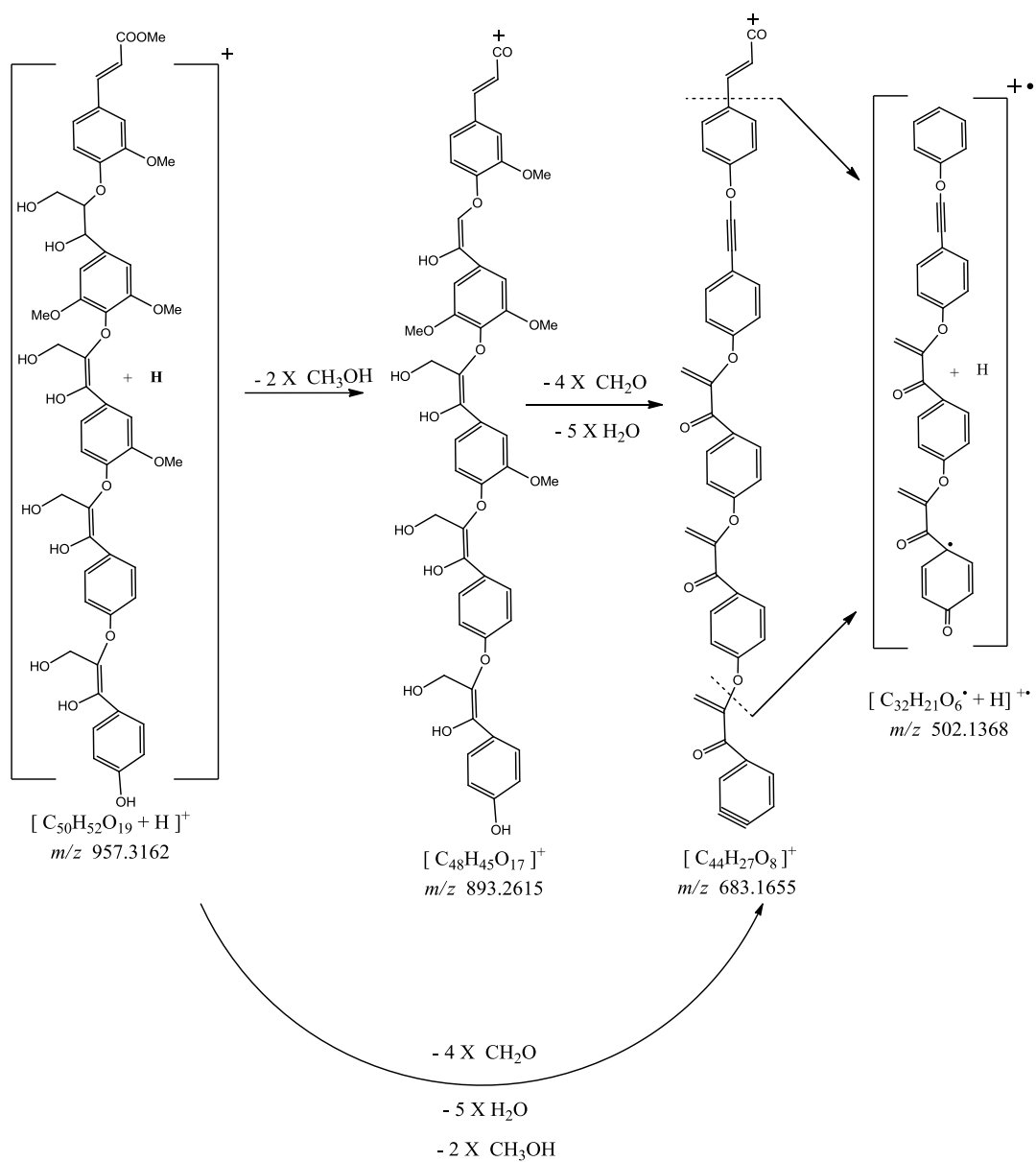
Scheme 5.4 (B). High-energy CID-MS/MS fragmentation pattern of the precursor distonic cation **2** at m/z 752.2636

The protonated molecule at m/z 957.3162 (Scheme 5.1) was assigned to have a molecular formula $[C_{50}H_{52}O_{19}+H]^+$, Double bond equivalent (DBE) = 25 and structure **(3)**. It was composed of a pentameric lignin oligomer formed of 5 aromatic rings, namely: H(8-O-4) H(8-O-4')G(8-O-4'')S(8-O-4''')G.

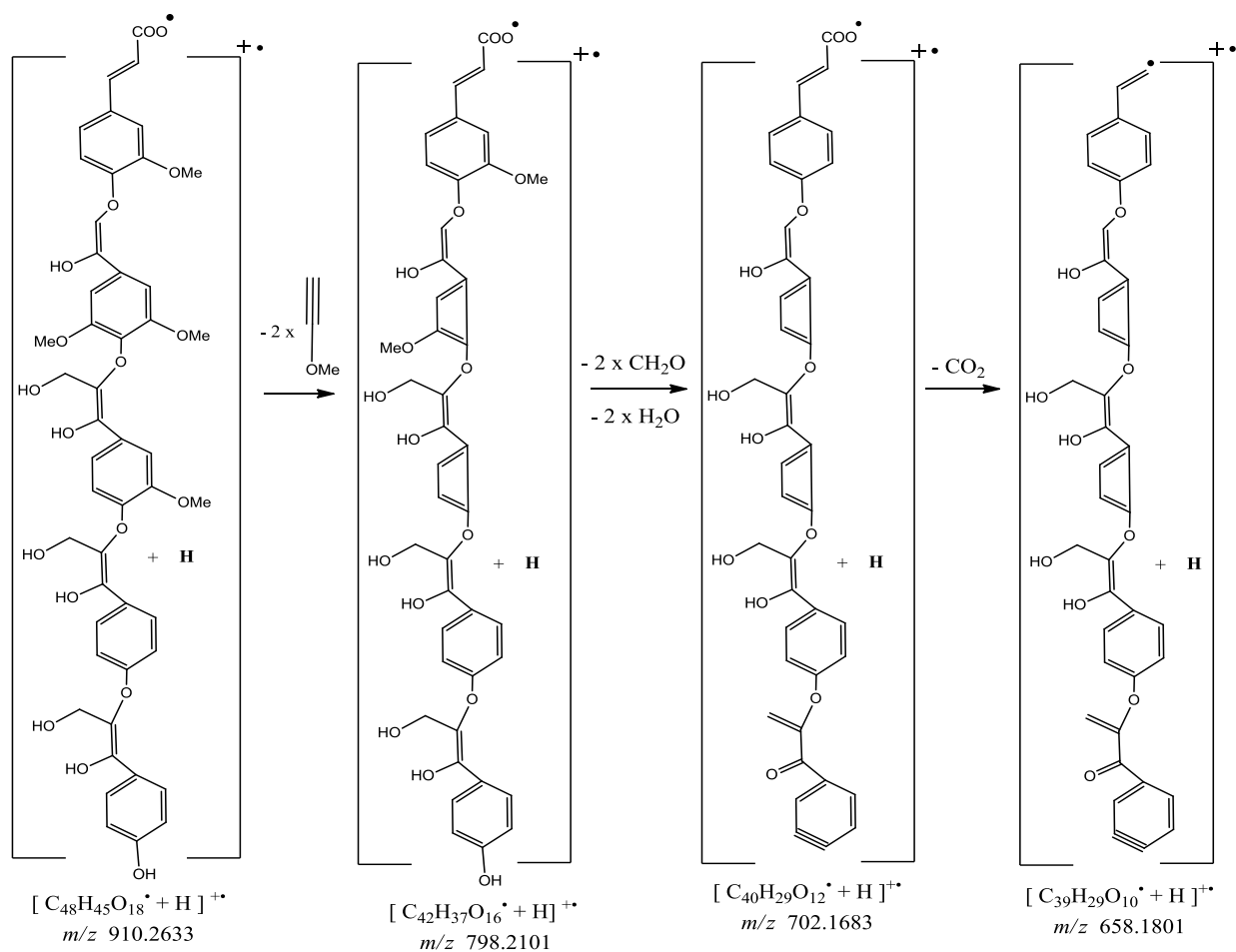
The product ion scan of the precursor ion **(3)** at m/z 957.3162 afforded the product ion at m/z 939.3014, created by the loss of a water molecule assigned to the molecular formula $[C_{50}H_{50}O_{18}+H]^+$. Moreover, the product ion at m/z 939.3014 can lose a molecule of formaldehyde to form the secondary product ion at m/z 909.2912 assigned as $[C_{49}H_{48}O_{17}+H]^+$. The precursor ion **(3)** can also CID fragments by of two consecutive molecules of methanol to affords the secondary product ion at m/z 893.2615 assigned as the $[C_{48}H_{45}O_{17}]^+$. Further consecutive losses of this product ion by four molecules of formaldehyde and five molecules of water afford the secondary product ion at m/z 683.1655, which was assigned as $[C_{44}H_{27}O_8]^+$. Moreover, the cleavage of the 8-O-4 bond of the first H residue of this latter secondary product ion, along with the cleavage of C1-C7 of the upper terminal H unit, affords the tetrameric product ion at m/z 502.1368, assigned as $[C_{32}H_{21}O_6 + H]^+$. It is noteworthy to mention, that the precursor protonated molecule **(3)** produced the dimeric product ion at m/z 347.1114, created by cleavage of the H(8-O-4)G bond which was assigned as the $[C_{18}H_{18}O_7+H]^+$ (Schemes 5.5A and B). The lower m/z values product ions are explained in scheme Schemes (5.5 A and B) . The remaining high-energy CID-MS/MS fragmentation patterns of the precursor ion **(3)** at m/z 957.3162, describing the formation of product ions having lower m/z are shown in schemes (5.5 C, D, and E).



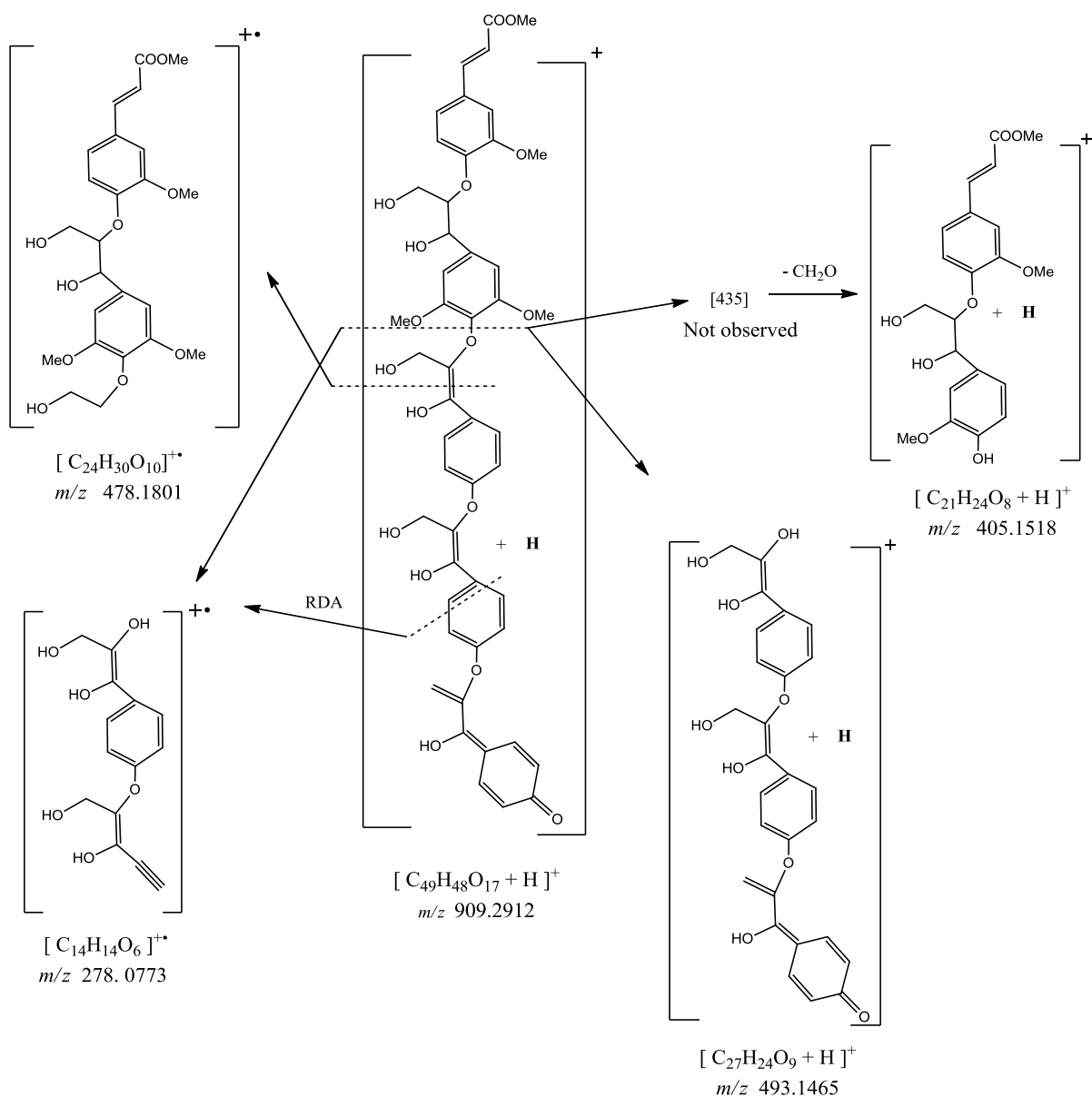
Scheme 5.5(A). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 3 at m/z 957.3162



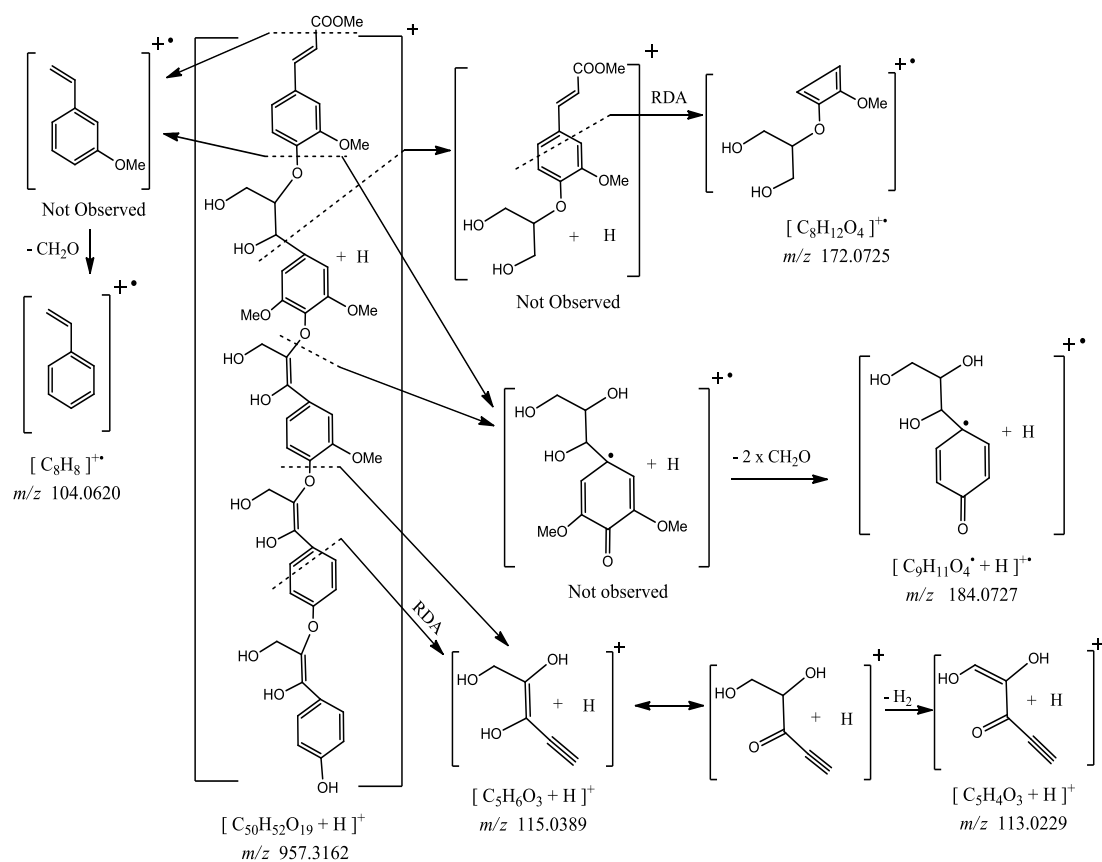
Scheme 5.5(B). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 3 at m/z 957.3162



Scheme 5.5(C). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 3 at m/z 957.3162



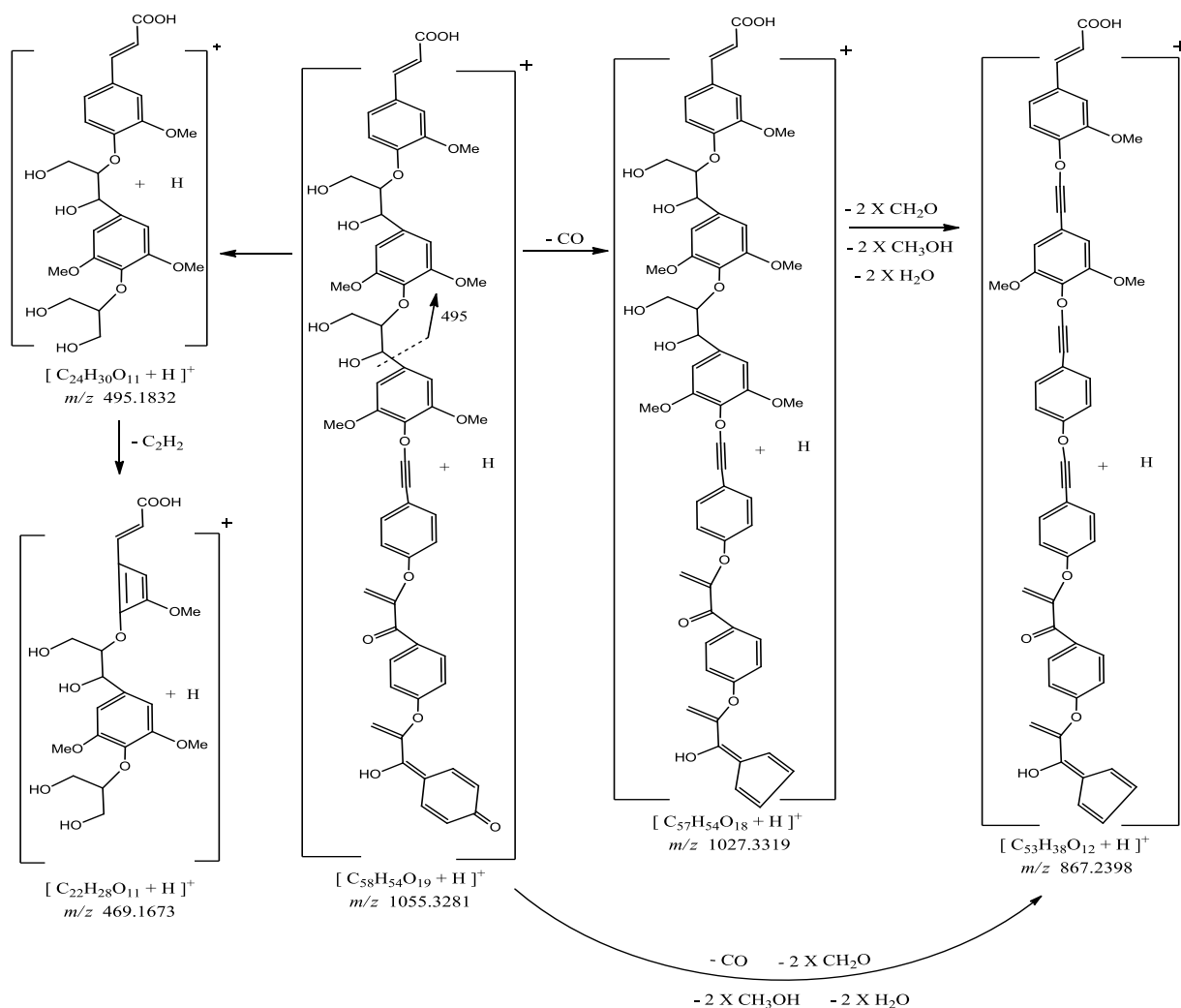
Scheme 5.5(D). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 3 at m/z 957.3162



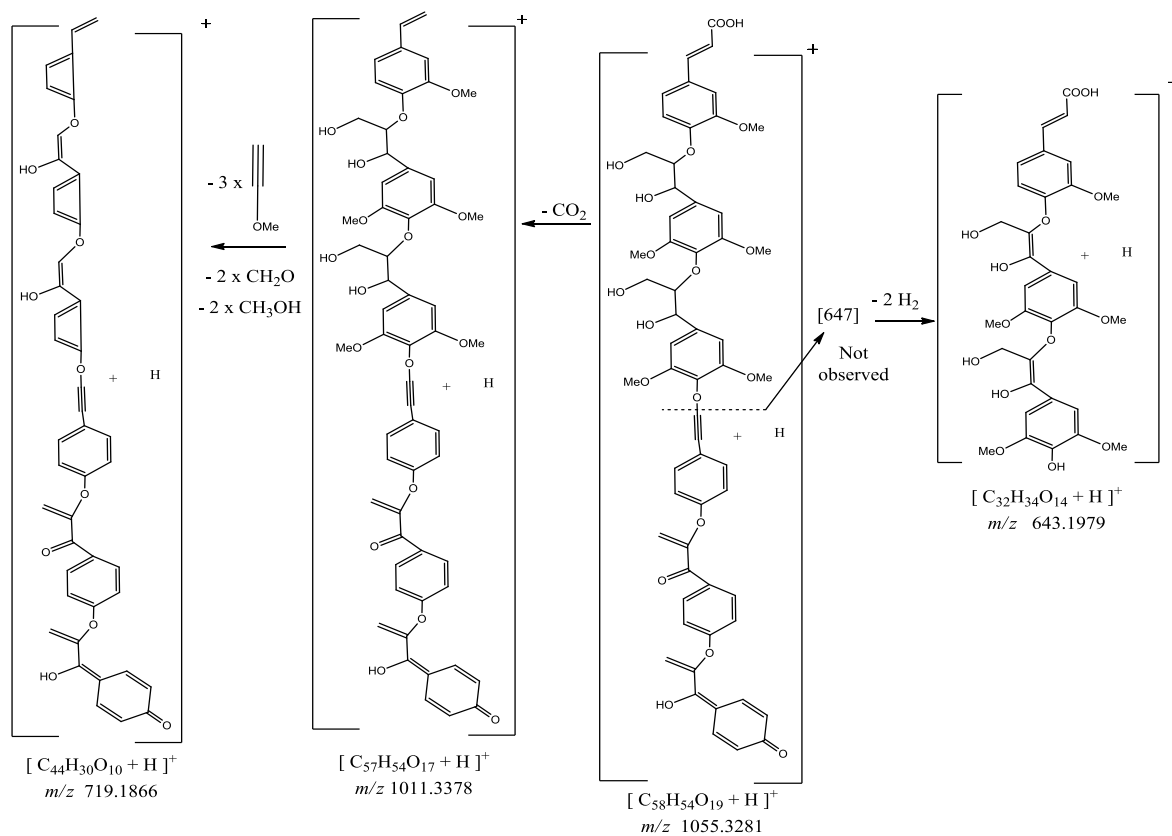
Scheme 5.5(E). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 3 at m/z 957.3162

The protonated molecule at m/z 1055.3281 (Scheme 5.2) was assigned as $[C_{58}H_{54}O_{19}+H]^+$ molecular formula, DBE = 32, and was composed of the hexameric unit formed by H(8-O-4)H(8-O-4')H(8-O-4'')S(8-O-4''')S(8-O-4''''')G. This protonated molecule was assigned as structure **(4)**. The product ion scan of this protonated molecule m/z 1055.3281 afforded the primary product ion at m/z 1027.3319 by loss of a molecule of carbon monoxide by ring contraction of the first H residue of this hexameric unit. This last product ion at m/z 1027.3319 loses by one concerted mechanism two molecules of water, two molecules of methanol and two molecules of formaldehyde, not necessary in that order, to afford the secondary product ion at m/z 867.2398 assigned as $[C_{53}H_{38}O_{12} + H]^+$.

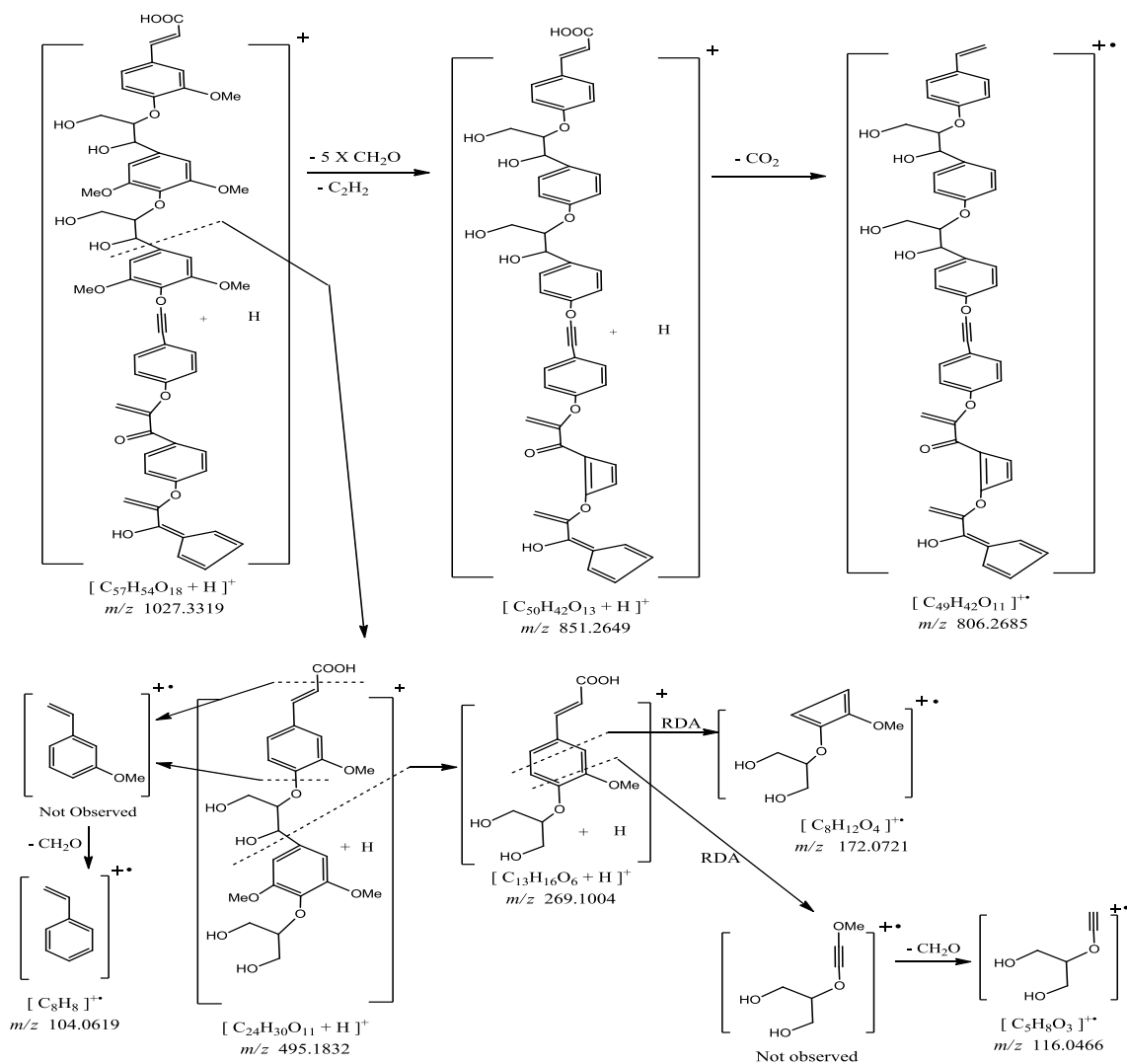
The CID-MS/MS cleavage of the precursor protonated molecule at m/z 1055.3281 (Scheme 5.6 A, B, and C) between the connecting two sinapyl residues affords the secondary product ion at m/z 495.1832 assigned as $[C_{24}H_{30}O_{11}+H]^+$. Finally, the presence of the carboxylic acid group was confirmed by the loss of CO_2 and H radical from the precursor ion at m/z 1055.3281 to afford the product ion at m/z 1011.3378 assigned as $[C_{57}H_{54}O_{17} + H]^+$. The lower m/z values product ions are explained in schemes (5.6 A and B). The remaining high-energy CID-MS/MS fragmentation pattern of the precursor ion m/z 1055.3281 describing the formation of product ions having lower m/z are shown in schemes (5.6 (C)).



Scheme 5.6(A). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 4 at m/z 1055.3281

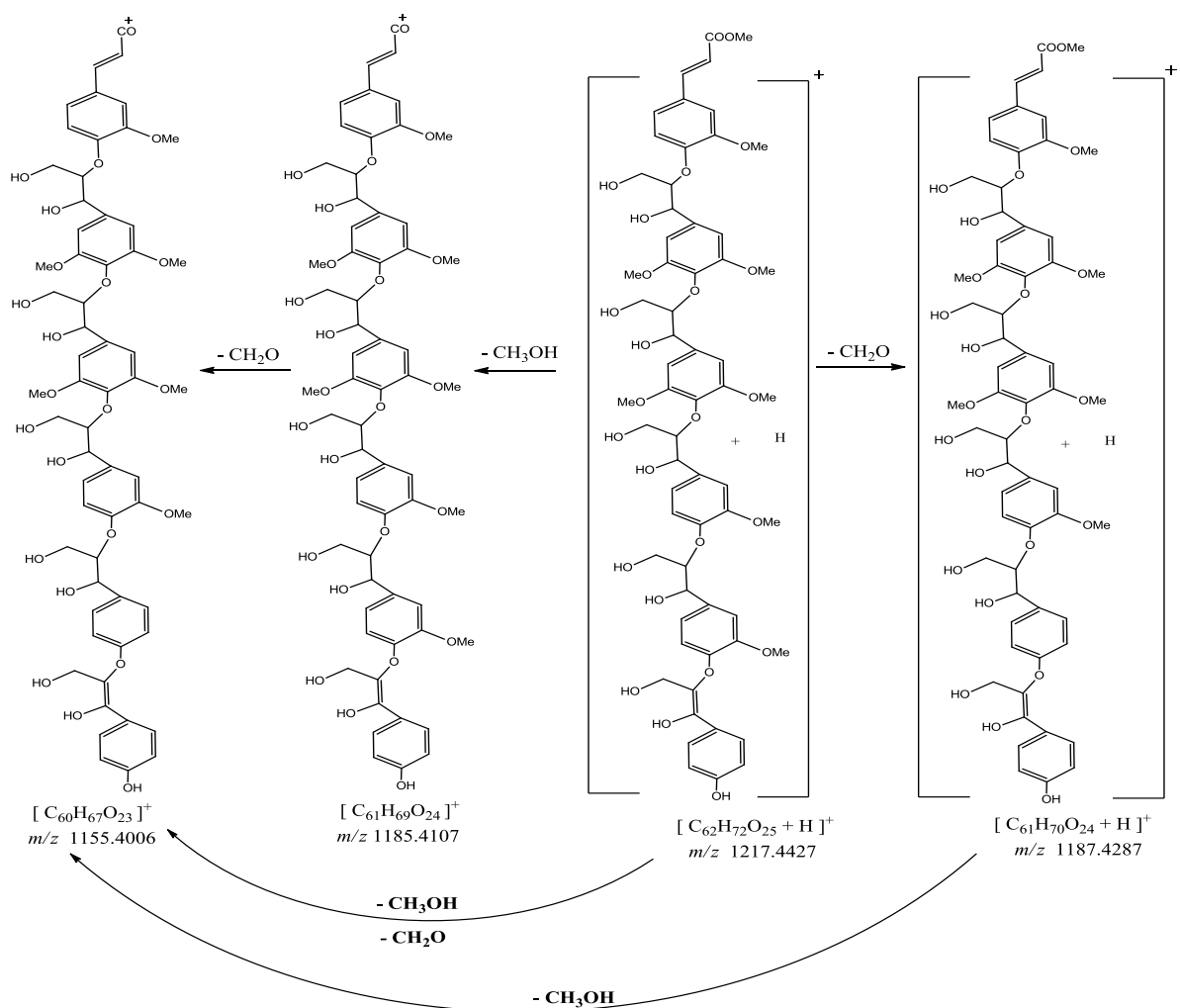


Scheme 5.6(B). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 4 at m/z 1055.3281

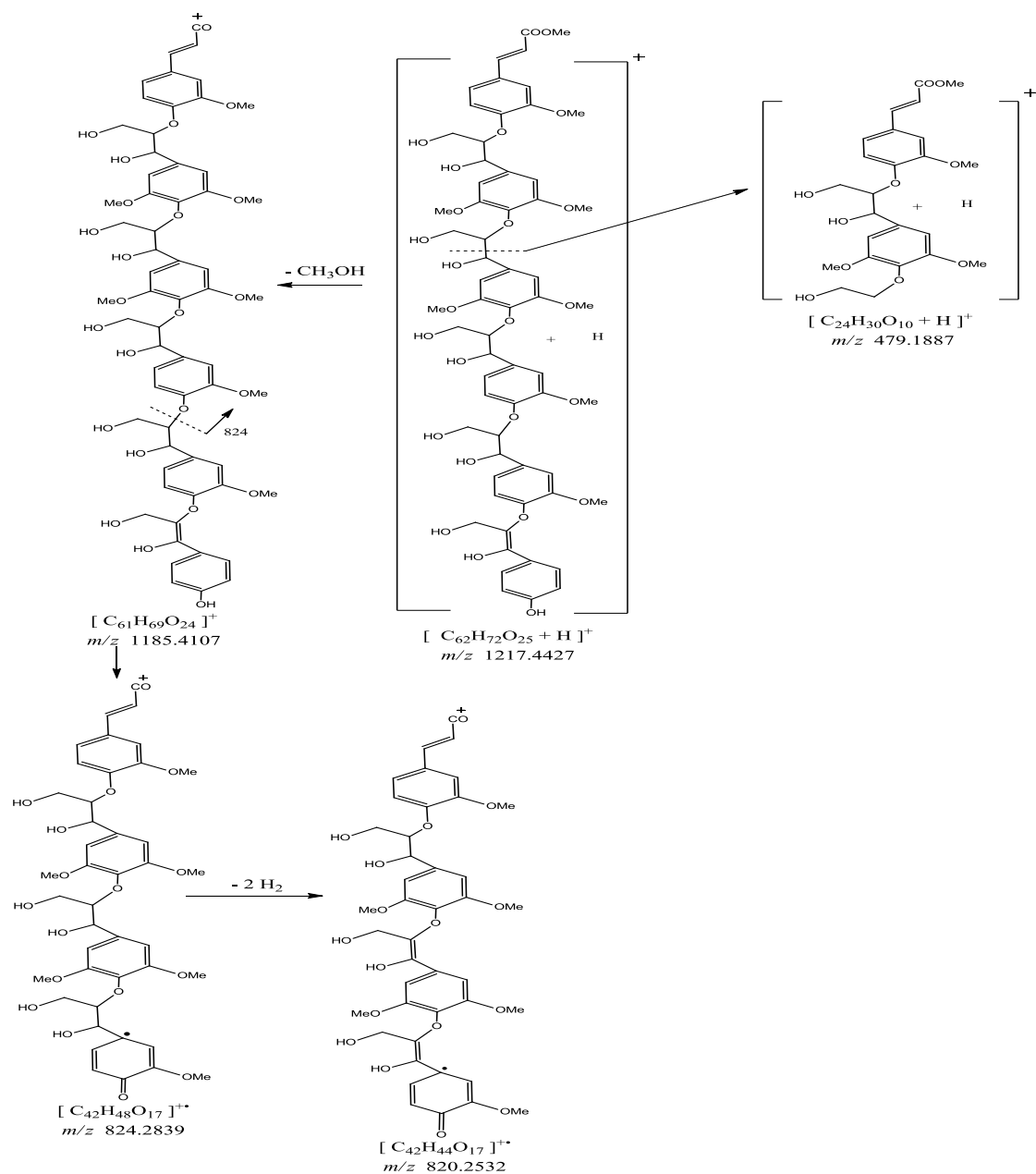


Scheme 5.6(C). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 5 at m/z 1055.3281

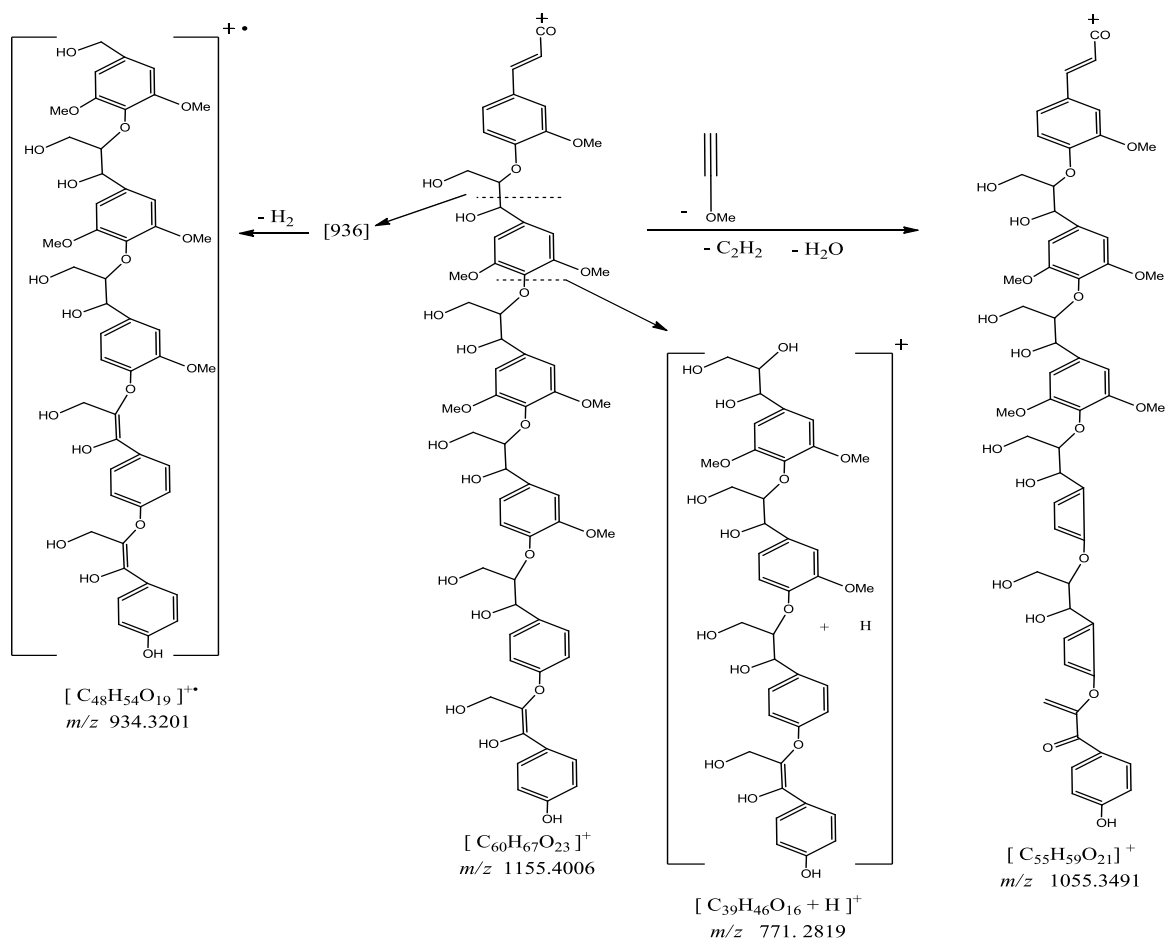
Lastly, the protonated molecule at m/z 1217.4427 (Scheme 5.2) was assigned as $[C_{62}H_{72}O_{25}+H]^+$, DBE = 27, and it was composed of the hexameric methyl ester oligomer formed of H(8-O-4)G(8-O-4')G(8-O-4'')S(8-O-4''')S(8-O-4''''')G. This protonated molecule was assigned the structure (5). The product ion scan of the precursor ion at m/z 1217.4427 afforded the primary product ion at m/z 1187.4287 by the loss of a molecule of formaldehyde, assigned as $[C_{61}H_{70}O_{24}+H]^+$. Similarly, loss of a molecule of methanol from this precursor ion gives the primary product ion at m/z 1185.4107 assigned as $[C_{61}H_{69}O_{24}]^+$. This latter product ion loses one molecule of formaldehyde to afford the secondary product ion at m/z 1155.4006 assigned as $[C_{60}H_{67}O_{23}]^+$. In addition, this same product ion at m/z 1185.4107 can experience a cleavage of G(8-O-4'')G bond to form the tetramer at m/z 824.2839 assigned as $[C_{42}H_{48}O_{17}]^+$. This latter product ion undergoes an oxidation by losing two molecules of hydrogen to afford the secondary product ion at m/z 820.2532. Finally, the cleavage of the precursor ion at m/z 1217.4427, between the two contiguous S(8-O-4''')S units, affords the diliginol product ion at m/z 479.1887, which was assigned as $[C_{24}H_{30}O_{10}+H]^+$. The high-energy CID-MS/MS fragmentation patterns of the precursor ion m/z 1217.4427 are shown in scheme (5.7. A and B).



Scheme 5.7. (A). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 5 at m/z 1217.4427



Scheme 5.7. (B). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule 5 at m/z 1217.4427



Scheme 5.7. (C). High-energy CID-MS/MS fragmentation pattern of the precursor protonated molecule (5) at m/z 1217.4427

To conclude, in this Chapter 5, we have commenced the first structural investigation of the VRL that showed that the Saudi date palm wood (*Phoenix dactylifera*), which is an excellent rich source of lignin. Furthermore, The MALD-TOF-MS analysis afforded six lignin oligomer protonated molecules including the following oligomers: HG, HGGG tetramer, HHGGG pentamer, HHHGGG hexamer and two HGGSSG hexamer. In addition, we noticed the formation of two distonic cations HGGG and HGSG.

On the basis of the total absence of structural information on the Saudi Date Palm Wood (SDPW) lignin in the literature and the complexity of this series of lignin oligomers, it is evident that the top-down lignomic new sequencing approach, allowed us to reveal this series of novel oligomers structures, that did not concur with the current knowledge of any lignin structures proposed (Chakar and Ragauskas, 2004; Grabber, 2005). To our knowledge, this is the first report on the structure of the lignin biomolecules extracted from Saudi date palm wood (*Phoenix dactylifera*) samples.

CHAPTER 6

Date palm as a new source of phenolic antioxidants as a source of woodsmoke in preparation of smoked salmon

This chapter represents a manuscript send to Journal of Food Biochemistry, and as such contains an introduction to wood smoke and its antioxidant properties. This manuscript is entitled “Date palm as a new source of phenolic antioxidants and in preparing smoked salmon”. Tasahil Albishi, Joseph Banoub, Adriano Costa De Camarguo, Fereidoon Shahidi.

6.1. Introduction

Smoked salmon is a highly valued product in Canada as in USA, and many other European countries. Most of the salmon offered to the general consumer are bought fresh and smoked within the country in which it is to be consumed. The majority of salmon smoked in Canada comes from the coast of British Columbia, with sockeye being the most important commercial species. Generally, the Wild Pacific salmon is available whole, or dressed into a range of cuts such as steaks, fillets and roasts and it is available in many value-added products as well, such as salmon caviar, hot and cold smoked salmon. Preservation of foods by exposure to wood smoke is probably the oldest form of food processing. The safety of smoked foods has been long established (Hilderbrand, 1992). Smoking is employed as a preservation method but, because of their volatile nature, natural compounds released during the process are also responsible for rendering a typical colour and flavour to smoked products such as meat and seafoods. Furthermore, it is also important to know the exact nature of smoke components in food products.

The chemistry of wood smoke is complex and includes carbonyl and phenolics among their functional groups. During pyrolysis of wood, its lignins produce the most important compounds in smoke, namely phenols and phenol ethers, typified by guaiacol (2-methoxyphenol), syringol (2,6-dimethoxyphenol) and their homologous derivatives. Studies on lignin have shown the presence of phenolic compounds such as ferulic, vanillic, syringic, and *p*-hydroxybenzoic acids (Catignani and Carter, 1982). Phenolic acids have also shown remarkable antimicrobial activity (de Camargo *et al.* 2017). Therefore, the presence of these compounds may contribute to the antioxidant and antimicrobial properties of lignin. Methoxyphenols were identified as prominent thermal degradation products of wood (Faix *et al.*, 1990) and as semi-volatile components of wood smoke particles (Hawthorne *et al.*, 1989). Antioxidant properties have also been demonstrated for several lignin-related methoxyphenols (Barclay *et al.*, 1997; Ogata *et al.*, 1997). Furthermore, different volatile compounds present in wood smoke may have large impact on the sensory quality of products (Wasserman, 1966). Therefore, it is likely that structural differences between components of the phenolic fraction result in major differences in the components' interactions with meat components such as their oxidative stability, which in turns may affect their flavour.

This study was aimed to investigate the feasibility of extending the options of woods to be employed in preparing smoked foods. To achieve this goal, the oxidative stability of smoked salmon was examined. Date palm wood (DPW) was used as the smoke feedstock, which was compared with the application of maple wood (MAW) which is a commercial wood smoke source. The potential antioxidant properties of both woods was screened by analyzing their total phenolic contents as well as their scavenging activity towards DPPH radical, both in their soluble and insoluble-bound forms. Solid-phase microextraction and gas chromatography–mass

spectrometry (SPME-GC/MS) were used to compare the volatile profile of both wood smokes and the effect of the process on the oxidative stability of smoked salmon was investigated by analysis of thiobarbituric acid reactive substances (TBARS). Colour changes, which are important to the product identity, were also evaluated. This study is helpful to gain better understanding of the influence of using a new type of wood for smoking during storage of smoked salmon by providing chemical and physical evidences for both fundamental and potential industrial benefit.

6.2. Experimental details

All experiments reported here have been explained in full details in Chapter 3.

6.3. Results and discussion

6.3.1 Screening the potential of date palm and maple wood as sources of phenolic antioxidants

Table 6.1 presents the total results of TPC and DRSC of DPW and MAW. It is not possible to anticipate whether soluble or insoluble-bound or both could participate in the generation of volatile phenolics present in smoke. Therefore, both forms were extracted and evaluated. The results showed that DPW had a higher TPC than MAW. Furthermore, regardless of the wood type, soluble phenolics made the highest contribution to the TPC. Zarzyński (2009) evaluated 25 wood species and reported that the wood's density correlated with the total quantity of phenolics. DRSC assay has been demonstrated to significantly and positively correlate with the antioxidant capacity of foods rich in unsaturated fatty acids (Arranz *et al.*, 2008). Therefore, this assay was chosen for evaluating the potential of DPW as a new source of phenolic antioxidants. DPW showed a higher ability in scavenging the DPPH radical (up to 9.6 fold) than that of MAW. In addition, soluble phenolics in both samples of wood showed a higher scavenging capacity

compared to the activity of the phenolics released from their insoluble-bound form, thus lending support to their contribution to the TPC data. Furthermore, a high positive correlation existed between TPC and DRSC assay ($r = 0.9947$) as shown in Figure 6.1, where the electron paramagnetic resonance (EPR) signal of phenolics extracted from DPW and MAW in scavenging the DPPH radical is displayed. Thus, it is possible to conclude that DPW could serve as a natural source of antioxidant as an alternative to MAW in different applications. In general, the results showed that DPW has a higher content of phenolic and flavonoids than MAW. Soluble and insoluble-bound phenolics for DPW were significantly different comparing their MAW, while bound flavonoids were not significantly different in bound form in their content. Research by Zarzynsk (2009) showed strong correlation between phenolic compounds and density of 12 tree species.

Table 6.1. Total phenolic contents (TPC) total flavonoid content (TFC), and DPPH radical scavenging capacity (DRSC) of soluble (S) and insoluble-bound (IB) phenolics of date palm wood (DPW) and maple wood (MAW)¹

Sample	TPC (mg GAE/g DE)		TFC (mg CA/gDE)		DRSC (mmoles TE/g of DE)	
	S	IB	S	IB	S	IB
DPW	73.48 ± 5.95a	21.63 ± 0.88a	13.61±0.31 ^a	1.96±0.041 ^a	25.59 ± 3.27a	6.50 ± 0.23a
MAW	52.27 ± 4.49b	16.77 ± 0.10b	7.08±0.96 ^b	0.68±0.036 ^a	19.01 ± 1.52b	0.68 ± 0.036a

¹Data represent mean values ± standard deviation (n = 3). Values followed by the same letters within a column are not significantly different (p > 0.05). GAE, gallic acid equivalents; TE, trolox equivalents; and DE, dried extract. n is a number of samples absorbed

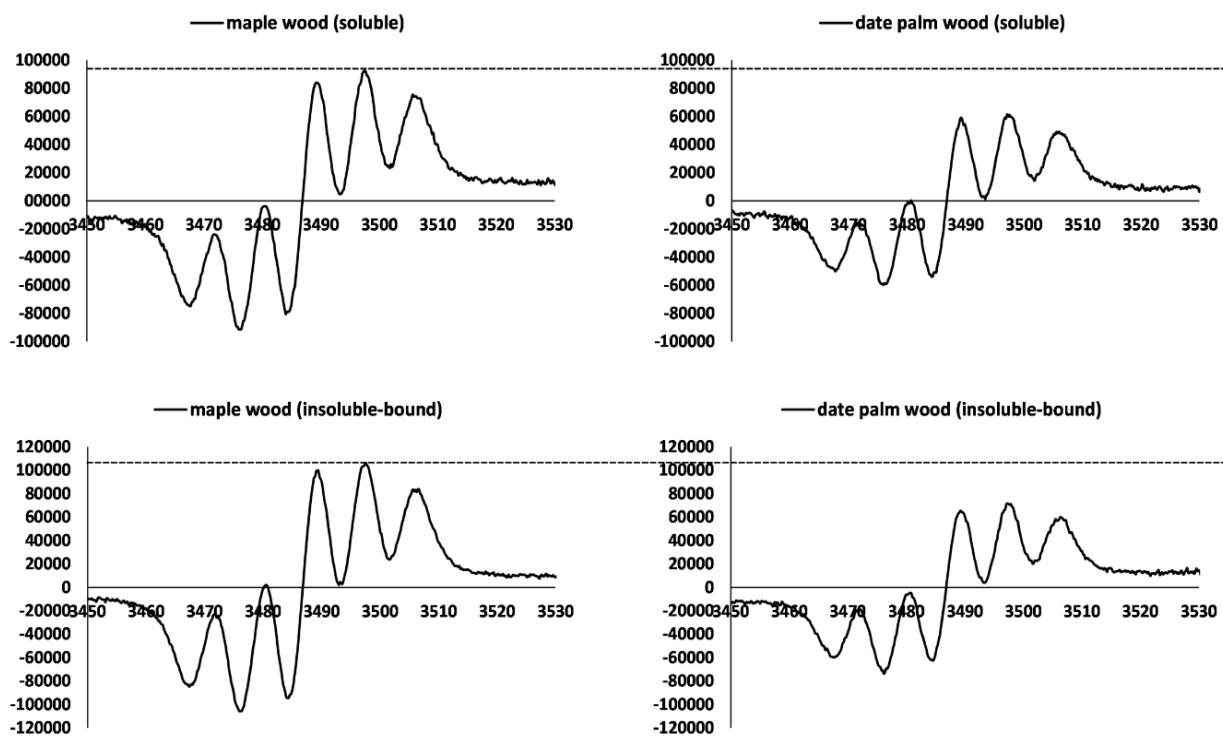


Figure 6.1. Electron paramagnetic resonance signals of soluble and insoluble-bound of phenolics of maple and date palm woods as evaluated by DPPH assay. The higher the EPR signal the lower the scavenging activity.

6.3.2. Application of date palm and maple wood as sources of volatile phenolic antioxidants in smoked fish model system

6.3.2.1. Volatile compounds of smoking wood

The aromatic character of smoke is influenced by the type of wood, method of generation and collection of smoke as well as combustion conditions. Lustre and Issenberg (1969) reported that only quantitative (not qualitative) differences existed in gas chromatograms of the phenolic fraction of hickory and mixed hardwood sawdust. However, these differences were not greater than those observed in phenolic fractions isolated from different batches. In the current study, smoke was produced from both DPW and MAW and the major volatile compounds produced from them were identified. The relative contents of volatile compounds obtained from the smoke generated from both woods are shown in Table 6.2. The results showed that smoke from DPW contained higher amounts of guaiacol, *p*-creosol, *p*-vinylguaiacol, syringol, *cis*- and *trans*-isoeugenol, methoxyeugenol, and syringaldehyde compared to those found in the smoke produced with MWt. The volatile profile was similar to those reported by Fujimaki *et al.* (1974). DPW also exhibited a more desirable smoky smell. Therefore, while both wood smokes contained almost the same volatile constituent, the compositions and/or their proportions varied slightly with the type of wood used. Regardless of the wood type, volatile compounds were mainly methoxyphenols, with the highest contribution from eugenol, followed by that of guaiacol and their corresponding derivatives. The aroma of eugenol (Adams, 1995) has been described as spicy, while green and fatty aroma were used to describe guaiacol (Högnadóttir and Rouseff, 2003). The phenolics detected were in good agreement with those reported in the literature (Fiddler *et al.*, 1967). Syringol, which showed eleven-fold higher concentration in DPW smoke, exhibits strong antioxidant effects (Kjällstrand and Petersson, 2001). It has also been claimed that syringol has a characteristic smoky aroma (Shahidi and Zhong, 2007).

Therefore, the differences in its concentration between the smoke produced from commercial and date palm woods may affect the sensory aspect of the smoked fish, although further confirmation of this point is required. Furthermore, such concentration differences may be explained by the existing differences in the component units of their lignins present in each wood type (Fujimaki *et al.*, 1974).

Table 6.2. Total area counts (x 10⁶) of identified smoking wood phenolic volatile compounds extracted by SPME with DVB–PDMS coated fibre

Compounds	Ki _{Exp}	Ki _{Lit}	Date palm	Maple wood	References
2-methoxyphenol (guaiacol)	1090	1091	3.14 ± 0.30a	1.44 ± 0.10b	Hognadottir & Rouseff (2003)
2-methoxy-4-methylphenol (<i>p</i> -creosol)	1190	1190	3.41 ± 0.45a	0.47 ± 0.12b	Adams (1995)
2-ethyl-4-methylphenol (<i>p</i> -ethylguaiacol)	1277	1275	1.98 ± 0.31a	2.06 ± 0.41a	Dalluge et al. (2002)
2-methoxy-4-vinylphenol (<i>p</i> -vinylguaiacol)	1307	1313	6.25 ± 0.93a	3.19 ± 0.28b	El-Sayed et al. (2005)
2,6-dimethoxyphenol (syringol)	1348	1348	4.14 ± 0.57a	0.36 ± 0.01b	Adams (1995)
4-allyl-2-methoxyphenol (eugenol)	1365	1356	5.99 ± 0.93a	5.39 ± 0.25a	Adams (1995)
2-methoxy-4-((Z)-1-propenyl)-phenol (<i>cis</i> -isoeugenol)	1410	1402	1.57 ± 0.21a	0.91 ± 0.13b	Adams (1995)
2-methoxy-4-((E)-1-propenyl)-phenol (<i>trans</i> -isoeugenol)	1461	1463	17.2 ± 1.77a	9.60 ± 1.70b	Dötterl et al. (2005)
4-allyl-2,6-dimethoxyphenol (methoxyeugenol)	1612	-	3.96 ± 0.06a	2.37 ± 0.54b	MS library*
4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)	1667	1656	3.48 ± 0.40a	2.31 ± 0.49b	Adams (1995)

Data represent the mean of duplicate analysis for each sample ± standard deviation. Means followed by the same letters within a column are not significantly different ($p > 0.05$). Volatile compounds were tentatively identified with the Wiley 9th Edition/NIST 2008 MS library and by comparing the experimental Kovats index (Ki_{Exp}) against the ones from the literature (Ki_{Lit}).

*methoxyeugenol was tentatively identified based on MS.

6.3.2.2. Oxidative stability as affected by smoking with date palm and maple woods

Lipid oxidation, which is especially important for salmon due to its high content of unsaturated fatty acids, is induced by thermal treatment. During lipid oxidation, MDA, a secondary oxidation product of fatty acids with 3 or more double bonds, along with other oxidation products, is formed. MDA reacts with 2- thiobarbituric acid (TBA) to form a pink TBA-MDA complex that is measured spectrophotometrically (Shahidi and Zhong, 2007). The TBARS of smoked fish samples stored at 4°C over 21 days are shown in Figure 6.2. Fresh salmon smoked with DPW (time zero) showed lower TBARS values (1.05 ± 0.00 MDA eq mg/kg) than that of the fish smoked with MAW (1.68 ± 0.02 MDA eq mg/kg) and this trend was held until the end of the experiment (7, 14, and days of storage). The oxidation of both samples increased during the storage; similar results were reported by Huong (2013) for smoked mackerel during storage. In the present study, after three weeks of storage the samples smoked with DPW showed 27.5% lower oxidation than those smoked with MAW, which is used for commercial purposes. Previous studies have demonstrated that cooked salmon devoid of antioxidants showed higher TBARS values compared to samples cooked in the presence of butylated hydroxyanisole (BHA) or phenolic extracts from different natural sources (Albishi *et al.*, 2013a; de Camargo *et al.*, 2017). Rosemary is rich in phenolic antioxidants such as carnosic acid and carnosol (Senanayake, 2018). According to da Silva *et al.* (2008), smoking increased the TBARS of smoked blue catfish, which was lower in rosemary extract-treated samples. Therefore, in the present study, it is possible to suggest that the heating process due to smoking also resulted in lipid oxidation in samples but, clearly, the phenolics released from DPW were more effective in decreasing their oxidation level compared to the standard maple wood. This contribution also focused on the identification and quantification of the volatile phenolics generated during smoking. A recent

study by Bjørnevik *et al.* (2018) reported the presence of phenolic compounds in fillets of Atlantic salmon subjected to different smoking procedures. Therefore, the lower TBARS of samples smoked with DPW may be related to a higher absorption of these phenolics into salmon fillet, which is supported by the higher TPC in the starting material as well as the higher contents of volatile phenolic compounds in the smoke, as determined by SPME-GC/MS. Findings of Mastelić *et al.* (2008) support the high antioxidant capacity of eugenol, a main component of wood smoke, as assessed by scavenging DPPH radical as well through the inhibition of lard oxidation using Rancimat. As mentioned already, eugenol and its corresponding derivatives made the highest contribution to smoke from both wood samples. However, a higher concentration of eugenol derivatives was detected in the smoke produced with DPW. Therefore, the higher oxidative stability of the salmon smoked with DPW may be, at least in part, explained by the high content of these phenolic compounds in the smoke. Furthermore, as emphasized recently (Granato *et al.*, 2018), it is also possible to demonstrate that total phenolics and the scavenging of DPPH may be good predictors in the screening of new type of wood to be employed in the smoking process of food.

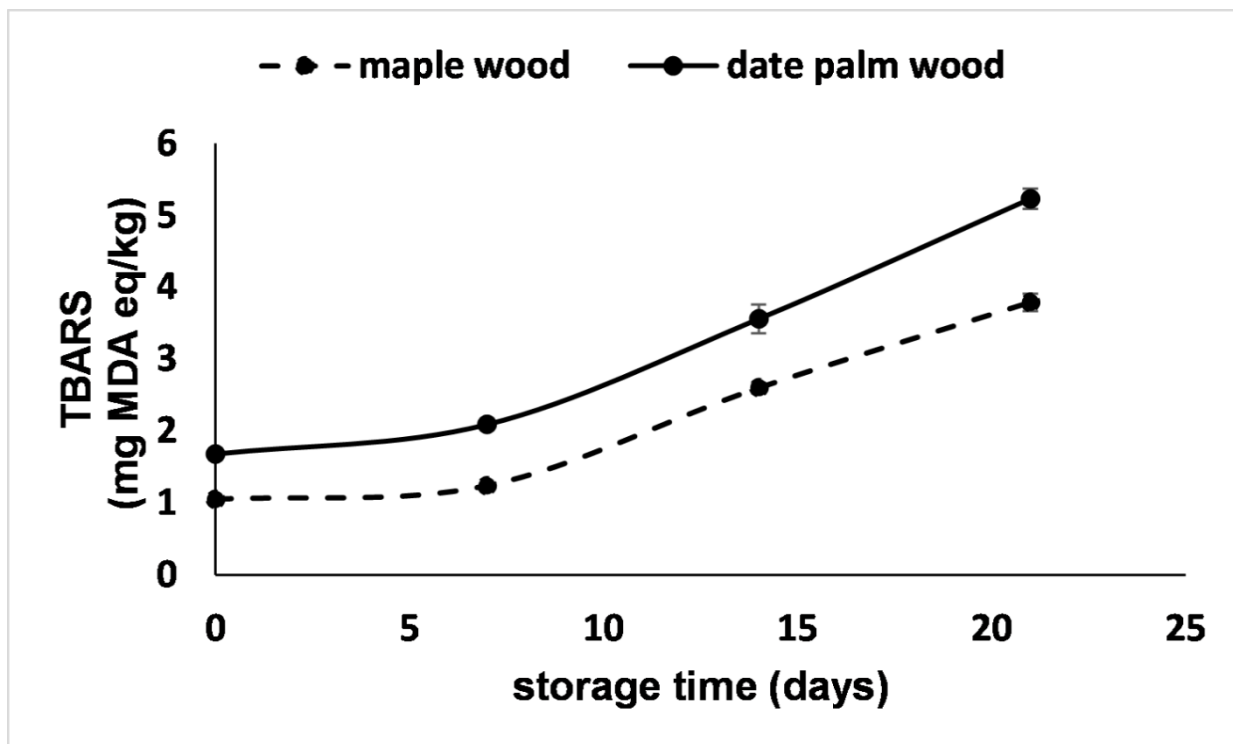


Figure 6.2. The TBARS of smoked fish samples stored at 4°C over 21 days. Data represent mean values \pm standard deviation ($n = 3$). No differences were found between the samples ($p > 0.05$). TBARS, thiobarbituric acid reactive substances; MDA eq, malondialdehyde equivalents.

6.3.3.3. Colour effects in smoked salmon

Several studies have demonstrated that colour, an important attribute related to the product identity, should be investigated in novel food products, as well as during food processing and long-term storage (da Silva *et al.*, 2014; de Toledo *et al.*, 2018; Selani *et al.*, 2016). Therefore, the colour of fillets subjected to smoking was measured for three different parts of the salmon, namely outside and middle portion as well as the skin. The results of the final products are shown in Table 6.3. After smoking (fresh samples), no significant difference in lightness (L^*) of the middle portion and skin was observed between DPW and MAW. In contrast, both fresh and stored samples smoked with DPW showed higher lightness values than that of MAW. The a^* value (redness) describes the intensity of the red colour (positive) of the smoked salmon. Significant difference in redness was observed between the two samples after smoking (fresh samples). However, this difference disappeared during the storage period. The b^* value (yellowness) describes the yellow (positive) intensity of smoked salmon. Regardless of the storage time and the part of the salmon, no difference in the b^* value existed between both smoked samples (DPW and MAW). The results presented here showed only a few differences between salmon fillets smoked with DPW and MAW. Furthermore, such minor differences, with exception of the lightness (outside portion), disappeared during storage. Therefore, DPW may be used as a substitute for MAW in smoked food preparation.

Table 6.3. Colour changes of fresh and stored salmon smoked with different types of wood

Sample	L*		a*		b*	
	fresh	21 days	fresh	21 days	fresh	21 days
outside						
DPW	46.81 ± 4.54 ^a	49.95 ± 6.81 ^a	22.07 ± 0.59 ^a	20.57 ± 1.82 ^a	23.11 ± 1.18 ^a	23.24 ± 1.38 ^a
MAW	35.88 ± 3.60 ^b	38.84 ± 1.73 ^b	17.08 ± 1.01 ^b	23.33 ± 0.44 ^a	20.59 ± 3.76 ^a	25.63 ± 6.23 ^a
middle						
DPW	52.30 ± 4.32 ^a	52.85 ± 2.07 ^a	20.99 ± 0.82 ^b	21.89 ± 0.90 ^a	15.46 ± 1.64 ^a	15.69 ± 1.49 ^a
MAW	52.10 ± 2.91 ^a	54.92 ± 3.93 ^a	23.02 ± 0.26 ^a	24.08 ± 0.49 ^a	15.99 ± 1.03 ^a	16.64 ± 0.91 ^a
skin						
DPW	26.86 ± 2.58 ^a	36.62 ± 6.79 ^a	17.06 ± 1.66 ^a	17.96 ± 2.54 ^a	5.49 ± 0.34 ^a	7.95 ± 0.57 ^a
MAW	28.70 ± 0.38 ^a	31.06 ± 4.96 ^a	7.18 ± 0.31 ^b	18.06 ± 0.94 ^a	2.67 ± 0.99 ^a	4.21 ± 0.17 ^a

L*, lightness; a*, redness; b*, yellowness; DPW, date palm wood; MAW, maple wood. Data represent mean values for each sample ± standard deviation (n = 3). Means followed by the same letters within a column part are not significantly different ($p > 0.05$). n is the number of samples observed.

CHAPTER 7

SUMMARY AND RECOMMENDATIONS

Six different types of wood (old date palm (OPW), seedling date palm (SPW), oak (OW), quebracho (QW), pine (PW), and banana (BW)) were examined for their bioavailability in antioxidant and biological activities. The results so obtained indicated that SPW and OPW had the highest amounts of phenolics, flavonoids, tannins, and lignin in both soluble (SP) and insoluble-bound (IBP) forms. The antioxidant activity of the selected woods was studied using the ability of their extracts to scavenge DPPH radical and their capacity to inhibit lipid peroxidation. The antioxidant activity and phenolic content of the SPW, OPW, OW, and QW determined by the DPPH and total phenols were higher than those of PW and BW wood samples. Analysis of wood extracts revealed a high number of polyphenols and flavonoids suggesting a possible role of these phytoconstituents in the antioxidant property. Moreover, the results clearly indicated that SPW had significant antioxidant activity.

This is the first study of the SP and IBP chemical composition, and antioxidant activities of date palm in particular and other selected woods in general. The results revealed that date palm wood in both types (SPW and OPW) contained several compounds. More than 70% of the components were identified as phenolic compounds which could be classified into phenolic acids, flavonoids, and lignin. Antioxidant activity assay results showed significant antioxidant activity comparable to other wood antioxidants. Considering the abundance of this wood as by-product and the influence of the chemical composition, new opportunities for the industry exist for developing diverse value-added products and explore potential applications from old date palm wood. In general, the antioxidant properties of the SPW, OPW, OW, and QW extracts were stronger than those of PW and BW. It is concluded that hardwoods are a rich source of natural

antioxidants and the extracts for this species can be considered as promising candidates for formulations of natural antioxidant supplements.

The second part of this study evaluated the antioxidant activity of and inhibition of lipid oxidation in smoked salmon. The results showed that the antioxidant activities of smoked SPW wood were contributed by the three major phenolic compounds, 2,6-dimethoxyphenol (syringol), 2-methoxyphenol (guaiacol), and 3,5-dimethoxy-4-hydroxytoluene. The chemical constituents of the date palm wood must be identified to maximize utilization in term of smoking process.

The analyses of the VRL of the Saudi date palm wood (*Phoenix dactylifera*) by MALDI-TOF-MS (+ ion mode) afforded a large number of protonated molecules that lead to complex spectra, that although were challenging to interpret but allowed us to obtain excellent blueprint of the different structures of the VRL oligomers. The high-energy tandem mass spectrometry analyses of these precursors' ions gave extremely complex spectra and allowed us to confirm the intricate proposed structures of this series of lignin oligomers. Therefore, we can say that the VRL of the Saudi date palm wood (SPW) is composed of units H, G and S in different proportions.

Based on the results obtained in this study, the future potential of date palm, oak, quebracho, pine, and banana wood extracts in the pharmaceutical and/or food industry exists and these need to be further assessed. In particular, further studies must be carried out on date palm wood due to a dearth of information about this renewable resource. Nowadays, consumers have an increasing interest in natural products, in food, supplements and cosmetic products. Given the inherent economic potential in the exploitation of natural resources in ecosystems, wood extracts can be used in cosmetic science in order to maintain the physiological balance of the human skin health. Furthermore, several by-products from the plant processing industry may be obtained that also helps the economy and addresses the waste disposal problems. Thus, natural plant extracts from

woods in this study can be used to obtain new natural antioxidants, preservatives, maximizing the utility of products currently underexploited or discarded.

Another important goal for future studies is in the food industry. During the past decades, plant-derived substances have attracted a great deal of attention mainly for their role in food preservation, particularly for the prevention of lipid oxidation. Being natural products, such as phenolic compounds that are extracted from plant, have been increasingly preferred over synthetic antioxidants for the reasons of being nontoxic and posing no health concerns. Furthermore, smoke produced from date palm may provide a viable alternative in the smoking of food products. Phenolics that are produced from smoked date palm wood have also been successfully produced and tested in salmon. Future research must be directed toward the screening of novel potential date palm woods as by-products for their phenolic composition and their antioxidative activity in various types of seafood, seafood products and muscle foods, in general. The use of extracts of woods examined here for stabilization and protection of food from spoilage also needs further studies.

Considering the abundance of this wood as a by-product of palm, new opportunities exist for developing diverse value-added products and explore potential applications of date palm wood in both food and nutraceutical areas. Accordingly, selected wood extracts, especially date palm, provide a safe, efficacious and cost-effective alternative to synthetic products. Further work is in progress to purify the wood extracts that gave the best biological activities in order to continue identifying the molecule(s) responsible for those desirable properties. Finally, the new findings in this research with respect to lignins in palm wood may provide opportunities for further research and development leading to potential industrial applications.

Publications

1. “Top–down lignomic MALDI-TOF-Tandem Mass spectrometry analysis of lignin oligomers extracted from Saudi date palm wood (SDPW)”. Tasahil Albishi, Abanoub Mikhael, Fereidoon Shahidi, Travis Fridgen, Joseph Banoub. *Rapid Commun. Mass Spectrom.*, 2018, 10-18-1002/rcm. Accepted and in press.
2. “ESI-Orbitrap-MS/MS and APPI-QqTOF-MS/MS structural elucidation of lignin oligomers extracted from Saudi date palm wood (SDPW)”. Tasahil Albishi, Abanoub Mikhael, Fereidoon Shahidi, Travis Fridgen, Joseph Banoub. *Rapid Commun. Mass Spectrom.* 2018, Submitted.
3. “Date palm as a new source of phenolic antioxidants and in preparing smoked salmon”. Tasahil Albishi, Joseph Banoub, Adriano Costa de Camargo, Fereidoon Shahidi. *Journal of Food Biochemistry*. 2018. JFBC-07-18-0593. Revised version submitted.

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